THE MECHANICAL PROPERTIES OF FISH MYOTOMAL MUSCLE

John Derek Atringham

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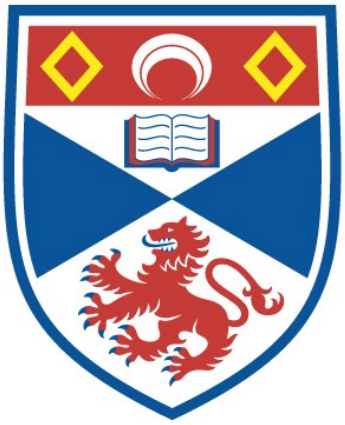

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A thesis submitted to the University of St. Andrews for the degree of Doctor of Philosophy

by

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DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. I.A. Johnston.

CERTIFICATE

I hereby certify that John D. Altringham has spent eleven terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1, 1967), and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

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SUMMARY

CHAPTER 1

A brief introduction is given to the structure, biochemistry and electrophysiological and mechanical properties of fish muscle.

CHAPTER 2

1. The neuromuscular end plates and preterminal axons of cod, Gadus morhua, fast myotomal muscle were stained for cholinesterase activity.
2. The number of end plates per fibre on superficial fast fibres (17.88 ± 2.13, mean ± 1 S.D.) was significantly higher than that of deep fast fibres (14.79 ± 2.48, $P << 0.001$). A small degree of multi-terminal innervation was noted. The end plates showed a great variety in structure and size.
3. Fast muscle contains fibres with a wide range of diameters (20-240 μm). However, no correlation was found between the number of end plates per fibre and fibre diameter.
4. A terminal branch of a given axon innervates fibres of a wide range of diameters.
5. It is concluded that fibres of different sizes have a similar pattern of innervation and are unlikely to represent functionally distinct fibre types.

CHAPTER 3

1. The force-velocity characteristics of threads of natural actomyosin, and purified component proteins, from dogfish fast and slow muscle, and

rabbit fast skeletal and cardiac muscle have been investigated. The apparatus used in this study is comprised of a lightweight aluminium lever pivoted near the centre. The position of the lever is monitored by means of a foil flag, attached to one end, passing across the path of a photodiode/LED assembly. The apparatus functions in both isotonic and isometric modes. In isometric, contraction causes a deflection of the flag, and the increased photodiode current fed back into a lightweight coil attached to the lever. This produces a restoring force, holding the thread at L_0. Isometric tension is proportional to the voltage across the coil (135 mV mg^{-1}). Switching the relay to the isotonic mode allows the thread to contract against a load determined by the pre-set current in the coil. Photodiode current is proportional to displacement (70 μm/100 mV). The switch to isotonic takes < 10 ms. Compliance is < 0.1 μm mg^{-1}, drift < 0.02 mg hr^{-1}.

2. Maximum isometric tensions were around 30-70 g cm^{-2}. The time taken to reach full tension after activation with ATP was 2-8 min.

3. Force-velocity curves obtained could be fitted to a linear form of Hill's equation (1938). In common with intact and skinned fibre studies, points below 0.7 P_0 were found to lie on a straight line.

4. Maximum contraction velocities were around 10^{-2} Ls^{-1}, 2-3 orders of magnitude lower than those of intact muscle fibres.

5. The relative velocities of the different thread types do not reflect those of the corresponding muscles, on the basis of measurements on intact fibres, and on measurements of actomyosin/myofibrillar ATPase activities.

6. It is concluded that filament formation, geometry and packing, and not differences in cross bridge cycling rates, largely determine the observed properties of actomyosin threads.

CHAPTER 4

A description of the apparatus used to study the isometric and isotonic properties of skinned fibres is given, together with the methods and protocol used in Chapters 5-7. The apparatus is a two-ended system for the independent measurement of tension and displacement. A silicon beam strain gauge is used to measure tension. The isotonic lever is pivoted at one end by attachment to the meter movement taken from a sensitive moving coil galvanometer. Displacement of the light balsa wood lever is monitored by a photodiode/LED assembly. During isometric contractions, the lever is held in position by the armature of a miniature relay. Activation of the relay allows the fibre to contract isotonically against a load determined by the current in the coil.

CHAPTER 5

1. The pCa-tension relationship of cod, Gadus morhua, and dogfish, Scyliorhinus canicula, fast and slow skinned fibres isolated from the myotomal muscles was investigated.

2. Maximum isometric tensions were 1.9 ± 0.12 (mean ± 1 S.E.) (fast) and 0.85 ± 0.10 (slow) for cod fibres, and 1.87 ± 0.09 (fast) and 0.84 ± 0.04 (slow) for dogfish (All values : kg cm^{-2}).

3. Sigmoid pCa-tension curves were obtained for all fibre types. Values for the half maximally activating [Ca^{2+}], and n, the minimum number of Ca^{2+} binding sites involved in activation, were calculated:

		n	[Ca^{2+}] half max (pCa)
Cod	fast	1.9	6.08
	slow	1.6	6.42
Dogfish	fast	3.5	6.41
	slow	3.2	6.50

Thus, the minimum number of Ca^{2+} binding sites in cod is two, in dogfish, four. In both fish, greater cooperativity is exhibited by the fast muscle.

4. The results are discussed in relation to those obtained from studies of other vertebrate, and invertebrate, preparations.

5. Dogfish muscle, in common with other elasmobranchs, contains high concentrations of osmoregulatory solutes, mainly in the form of urea and TMAO. The effect of these solutes on P_0 have been investigated. Urea significantly inhibited tension generation at 8°C (P_0 decreased by 20%). This inhibition was almost totally abolished by the addition of physiological concentrations of TMAO. These results further support the theory that methylamine compounds present in elasmobranch tissues counteract the protein destabilising effects of urea.

CHAPTER 6

1. Force-velocity curves were derived from fast and slow skinned fibres isolated from cod and dogfish myotomal muscles.

2. The extrapolated V_{max} and the constants a and b were calculated from a linear form of Hill's equation:

		V_{max} (Ls^{-1})	a/P_0	b (Ls^{-1})
Cod	fast	1.01	0.21	0.21
	slow	0.53	0.28	0.21
Dogfish	fast	2.34	0.06	0.14
	slow	0.68	0.19	0.13

3. These results are discussed with reference to previous studies of the P-V relationship in amphibian and mammalian muscle. The relationship between a/P_0 and efficiency, and its bearing on the present results is discussed.

4. Velocity transients showed a small departure from linearity in all experiments, with velocity decreasing continuously during release (usually < 25% over the first 250 ms after release). This is a feature common to many previous experiments on skinned and intact fibres. The decrease in velocity during release was particularly marked in cod slow fibres. Similar results have been reported in amphibian slow muscle.

CHAPTER 7

1. Contraction velocity at low loads was studied in dogfish fast fibres during maximal and submaximal activations.

2. The velocity of contraction during the second 50 ms interval after the onset of release was reduced significantly at low $[Ca^{2+}]$. The shape of the velocity transient was found to be dependent on $[Ca^{2+}]$. The rate of decrease of velocity was greater in submaximal than in maximal activations.

3. A brief review of previous studies on the dependence of V_{max} on $[Ca^{2+}]$ is given.

4. The results are discussed in the light of recent evidence for length dependent changes in the contractile system. Possible mechanisms for a Ca^{2+} and length dependent inactivation process are considered.

CHAPTER 8

The major outstanding problems are stated, and suggestions are made for further work which may give a greater insight into the molecular events underlying contraction in fish muscle.

CHAPTER 1

INTRODUCTION

THE LOCOMOTORY MUSCLES OF FISH

Swimming in fish is achieved by undulations of the segmental (myotomal) muscle, which pass backwards along the body, and by the use of the paired and unpaired fins. This review will deal entirely with studies on myotomal muscle. The myotomal muscles constitute 40-60% of the total body mass (see Bone, 1978a). Because of the high density of water, relative to air, pressure drag due to inertial forces is high, and much effort is also wasted in pushing against a yielding medium, creating eddies (see Webb, 1978, and Magnuson, 1978, for reviews on hydrodynamics in relation to body form). The enormous bulk of the body musculature allows a fish to generate sufficient power to swim rapidly in such a medium, and the buoyancy imparted by water excludes the need for economy in weight. In terrestrial locomotion (in general) power requirements increase linearly with the speed of locomotion. In contrast, because of the different constraints imposed by water in aquatic locomotion, power requirements rise as a cube function of swimming speed (see Webb, 1975). For this reason, the bulk of the musculature is made up of fast glycolytic fibres, used during short periods of burst swimming, which can develop power rapidly, and are to a large extent independent of the circulation (as will be discussed below, this is an oversimplification of the situation in many teleosts).

The myotomes of fish are complex in shape, and there is a phylogenetic increase in complexity, from the V shape of amphioxus to the deep W shape of the teleosts. In the more primitive fish, the unequal length of the V shaped myotome is thought to be a mechanism for the

prevention of dorsoventral flexion during swimming (Bone, 1978a). The complex myotomal shapes of higher fish have been investigated by Alexander (1969). In elasmobranchs and teleosts, the myotomes form a series of stacked, overlapping cones, pointing anteriorly and posteriorly. The fibres in each myotome insert into a connective tissue sheath, the myoseptum, which separates adjacent myotomes. The slow fibres, lying under the skin, run approximately parallel to the long axis. The arrangement of the fast fibres is complex, and differs not only between classes, but in different parts of the same fish. For example, the pattern found in Scyliorhinus and other elasmobranchs is very different from that of the more advanced teleosts, but in the caudal region of teleosts the arrangement reverts to that of the Selachians. In general, the superficial fast fibres run parallel to the long axis of the body, but with increasing depth, their orientation changes, and deep fibres may make angles of up to 35^{o} with the vertebral column. Fibre orientation through successive myotomes describes a series of helices, with their axes running parallel to the long axis of the body. After a careful analysis of these arrangements, Alexander concluded that during the body flexures associated with swimming, each fibre would shorten by the same amount, irrespective of its position. This has obvious and important implications. All muscles have lengths for optimum tension generation, and rates of contraction for optimum power output. The fast muscle of fish is presumably adapted for a high power output, and this will be maximised if all fibres operate under the same constraints of length and velocity.

Fibre Types

A characteristic feature of fish muscle is the anatomical separation of the various fibre types. This arrangement in Torpedo led to one of the first descriptions of vertebrate fibre types by Lorenzini in

1678. Although small differences are seen in the arrangement of these fibres within the trunk, the basic pattern is essentially the same in all fish. The jawless groups, the Acrania and Agnatha, will not be discussed, but an excellent review has been made by Bone (1978a). In most fish the slow fibres are found as a superficial layer just beneath the skin. They may circumvent the whole of the trunk as in dogfish (Bone, 1966), or form a more discrete wedge under the lateral line as in the brook trout, Salvelinus fontanalis (Johnston and Moon, 1980b). Exceptions to this rule include the skipjack tuna (Katsuwonus pelamis), which has an internalised muscle mass. In this case, it is associated with a vascular counter-current heat exchange system used to maintain elevated brain and slow muscle temperatures (Sharp and Pirages, 1978). The properties of this muscle will be discussed below. Slow muscle constitutes between 0.5-29% of the myotomal muscle mass (Greer-Walker and Pull, 1975). The proportion of slow muscle is related to the activity level of the fish, being highest in pelagic fish, and lowest in sedentary bottom dwellers and those fish which swim primarily by fin movements. Beneath the slow fibres, and making up the bulk of the musculature, is the fast muscle mass. In most species, both slow and fast fibres can be subdivided on the basis of histochemical, ultrastructural and biochemical differences. On these criteria, the transition from one fibre type to another is in fact not always very abrupt; the tendency is rather towards a gradual transition from superficial slow fibres to deep fast fibres.

Biochemical and Histochemical Studies

This transition is illustrated nicely in the dogfish, Scyliorhinus canicula, which has been characterised by Bone (1966, 1978a). There is a single interrupted outer layer of large diameter superficial fibres, with negative staining for succinic dehydrogenase activity (SDHase), a

marker for the TCA cycle, and therefore an indication of the aerobic capacity. These fibres also have a low staining for myofibrillar ATPase activity (Bone and Chubb, 1978). Bárány (1967) has shown a positive correlation between biochemical measurements of myofibrillar ATPase activity and contraction velocity. Biochemical and histochemical techniques are therefore used as an indirect estimate of the speed of contraction. Beneath this superficial layer are small diameter fibres with a high SDHase staining and low myofibrillar ATPase activity. The deepest 2-3 layers of these slow oxidative fibres can be distinguished on the basis of a slightly higher myofibrillar ATPase activity and a less intense SDHase reaction. The bulk of the musculature is composed of large diameter fast fibres with a low oxidative capacity. The outer 2-3 layers of this group can be distinguished on the basis of a smaller size, more positive SDHase reaction, and a more abundant capillary supply (Bone, 1978a). Biochemical measurements of myofibrillar ATPase activity (Bone and Johnston, 1980) correlate well with the histochemical results. With one or two exceptions, which will be discussed below, this pattern, with varying degrees of complexity, is seen in all elasmobranchs and teleosts. In the brook trout, Salvelinus fontinalis (Johnston and Moon, 1980b), Atlantic mackerel, Scomber scombrus (Bone, 1978b) and the rainbow trout, Salmo gairdneri (Johnston et al., 1975), we have the simplest arrangement, with only two fibre types, easily distinguishable on the basis of aerobic capacity and biochemical and histochemical myofibrillar ATPase activity. Fast fibres commonly show a wide, continuous distribution of fibre diameter, which may overlap with slow fibre size at the lower end.

In some species, an intermediate fibre type is found between the fast and slow muscle zones, and these are sometimes referred to as pink fibres on the basis of their intermediate colour. In the carp, Carrasius carrasius, the pink fibres constitute 10% of the myotomal

muscle, a greater proportion than the slow fibres (Johnston et al., 1974; Mosse and Hudson, 1977). Pink fibres, on the basis of biochemical studies, have aerobic enzyme and myofibrillar ATPase activities between the fast and slow fibres, and a myosin light chain composition characteristic of fast fibres (Johnston et al., 1977). These fibres therefore probably correspond to an aerobic fast muscle type, as has been described for other vertebrates (Goldspink, 1977).

A more complete functional picture of the various fibre types can be gained from ultrastructural studies. Table 1:1 summarises the more important points revealed by recent quantitative work. The most noticeable feature to arise from these studies is the high mitochondrial content of slow fibres, which approaches that found in mammalian ventricular muscle (Bossen et al., 1975). Mitochondrial content in fast fibres is very much lower. An interesting point not brought out in the table is the higher mitochondrial fractional volume of teleost fast fibres in comparison to elasmobranchs. Take, for example, the value of 8.9% for brook trout (Johnston and Moon, 1981) and those of 1 and 0.5% for the sharks Galeus malestomus and Etmopterus spinax (Kryvi, 1978). This small but significant difference is a reflection of the somewhat different roles of the fast fibres in the two classes, which will be discussed below when innervation and fibre recruitment are covered.

Both the sarcoplasmic reticulum (S.R.) and the T-system occupy greater fractional volumes in fast muscle than in slow, as in other vertebrates (see, e.g., Flitney, 1971; Totland, 1976, for amphibian data, Nag, 1972; Kryvi, 1978, for fish data). However, the absolute volumes occupied by S.R. in fish slow muscle is higher than in amphibians, approaching that of twitch fibres (Johnston, 1980b). This may be explained by the active locomotory role of fish slow fibres compared to the postural function attributed to amphibian slow fibres.

Myofibrillar fractional volume in fast fibres is higher than in

slow, and myofibrillar packing is somewhat more regular. An unusual feature of small diameter fast fibres in teleosts is the elongation of the peripheral myofibrils to give a series of radial arms, resembling the spokes of a wheel.

As predicted from the high aerobic capacity of the slow fibres, they have an abundant supply of capillaries. Slow fibres (with the exception of tuna (Guppy et al., 1979)) also have the more abundant store of both lipid and glycogen (Love, 1980), but quite large stores of glycogen may be found in fast fibres.

Other Fibre Types

The existence of an internalised slow muscle mass in the skipjack tuna, Katsuwonus pelamis, and other Scombridae has been noted above. These slow fibres are particularly well adapted to the demands placed on them by the pelagic existence of the fish, having ten times more mitochondria than the superficial slow fibres and a more highly developed capillary bed (Bone, 1978b). Johnston and Tota (1974) found myofibrillar ATPase activity in the deep slow fibres of the Atlantic blue fin tuna, Thunnus thunnus, to be twice as high as analogous muscles of less active fish. Tuna appear to have a significant capacity for aerobic glucose metabolism in both fast and slow muscles (Guppy et al., 1979).

A "mosaic" arrangement within the fast muscle mass has been described in cod, Gadus morhua (Greer-Walker, 1970), in the anglers, Lophius (Bone and Chubb, 1978), and in the rainbow trout, Salmo gairdneri (Johnston et al., 1975). Smaller diameter "red" fibres with ~~less~~ more positive SDHase staining and a ~~higher~~ lower (histochemical) myofibrillar ATPase activity are found scattered among the large diameter fast fibres. It was thought that these fibres were distinct fibre types, but the general concensus now is that they represent different stages in growth (Johnston et al.,

1975; Korneliussen et al., 1978; Johnston and Moon, 1980b). However, there is a trend for smaller diameter fast fibres to have higher mitochondrial contents than large ones (Johnston and Moon, 1981). In contrast to most other vertebrates, fibre number increases throughout life in fish (Greer-Walker, 1970).

Innervation

All fish slow fibres are multiply innervated by small diameter myelinated axons, terminating in en grappe end plates (Barets, 1961; Bone, 1966, 1970; Best and Bone, 1973). In elasmobranchs and teleosts at least two axons innervate each slow fibre (Bone, 1978a). In elasmobranchs such as the dogfish, subjunctional folds are present under the terminations (Bone, 1972). Subjunctional folds are not found in teleosts (Nishihara, 1967). Acetylcholinesterase has been demonstrated at all slow fibre terminations so far investigated (Bone, 1978a).

The fast fibres of elasmobranchs and the taxonomically primitive teleosts are focally innervated, and in the case of the elasmobranchs nearly always at one myoseptal end (Bone, 1978a). Two axons form a single end plate and, in some elasmobranchs, two types of terminal can be recognised (Bone, 1972). Cholinergic vesicles have been identified in one type, the other containing somewhat larger vesicles, but as yet only acetylcholinesterase has been identified in the subjunctional folds (Pecot-Dechavassine, 1961). One exception to the above generalisation is Torpedo, in which the superficial fibres receive basket-like terminations in the mid-region (Bone, 1964). The innervation of the lower teleosts, although not extensively studied, appears to resemble that of elasmobranchs (Barets, 1961; Bone, 1964, 1970).

The fast muscles of taxonomically advanced teleosts receive a multiple, distributed innervation (Takeuchi, 1959; Barets, 1961; Bone, 1964). This pattern of fast muscle innervation is almost unique among

the vertebrates, and there is evidence to suggest that it has evolved on at least eight separate occasions in fish (D. Ono - personal communication). Branches of the spinal nerve run in the myosepta, and fan out as a diffuse network over the myotome (Barets, 1961; Bone, 1964). In an investigation of the long horned sculpin, Myoxocephalus scorpius, Hudson (1969) found that each fibre receives 2-5 axons from each of five spinal nerves, the number of terminations ranging from 8-22, an average of 0.7 mm apart. The end plate formations show enormous structural variety (Barets, 1961; Hudson, 1969).

Electrophysiology

With respect to their innervation and electrophysiological properties, the slow fibres of fish resemble the true slow fibres of amphibia, depolarising pulses eliciting junction potentials (Hagiwara and Takahashi, 1967; Stanfield, 1972). Stanfield, however, found sufficiently large inward Na^+ currents in 8 out of 27 of the dogfish slow fibres to suggest that they may be capable of generating action potentials. Similarity to the amphibian pattern extends to the fast fibres of focally innervated species. In all examples studied to date, typical overshooting, propagated action potentials are elicited in response to depolarisation (Hagiwara and Takahashi, 1967).

The electrophysiological properties of the polyneuronally innervated fast fibres of teleosts have been investigated by Barets (1961), Hidaka and Toida (1969), and Hudson (1969). Hudson found that electrical stimulation of the spinal nerves of Myoxocephalus scorpius produced either overshooting action potentials, accompanied by a fast twitch, or junction potentials associated with local graded contractions. Low intensity stimulation produced summating junction potentials to produce action potentials. In fresh preparations an action potential could arise from a single 15-35 mV junction potential.

Mechanical Properties

Few studies of the mechanical properties of myotomal muscle have been made. The complex arrangement of myotomal fibres makes the dissection of a preparation in which all fibres are parallel rather difficult, and the myoseptal insertions are unsuitable for attachment to the recording devices. Studies have therefore been restricted mainly to fin and jaw muscles (e.g., Hidaka and Toida, 1969; Yamamoto, 1972). Bone and Johnston (1980) looked at fast and slow fibre bundles isolated from dogfish myotomes. Under supramaximal stimulation, fast fibres gave a twitch (half time to peak tension, $t_{1/2}$ = 20 ms), and slow fibres a more prolonged contraction ($t_{1/2}$ = 100 ms), with fused tetani produced at > 8 Hz in both fibre types. Very different characteristics have been reported for polyneuronally innervated fast fibre bundles isolated from Tilapia adductor operculi muscles (Flitney and Johnston, 1979). Both fast and slow fibres produced graded fused tetani, with maximum tensions at > 250 Hz. Slow fibres did not respond to stimulation frequencies < 5-10 Hz. V_{max} in fast fibres was 2.6 Ls^{-1}, 1.5 Ls^{-1} in slow fibres. However, the rate of tension development was found to be critically dependent upon stimulation frequency and at 200 Hz was 6.5 times greater in fast fibres than in slow. A similar dependence of the rate of tension development on stimulation frequency was found in polyneuronally innervated cod fast fibres, in a comparison with the focally innervated fast fibres of the cuckoo ray, Raija neavus (Johnston, 1980a). Tetanic fusion frequencies were 5-10 Hz in the ray, 40-50 in cod, and maximal tensions were reached at 20 and > 200 Hz respectively. Since teleost fast fibres are thought to operate almost isometrically during swimming, it may be that the rate of tension development, and of body flexure, will be determined as much by membrane characteristics as by the intrinsic maximum contraction velocity.

Parvalbumins

Free cytoplasmic Ca^{2+} binding proteins, parvalbumins, are found in all vertebrate fast muscles. Only in fish fast muscles do they occur in high concentration, constituting approximately 15% of the soluble proteins (Le Peuch et al., 1978). A high degree of homology has been noted between parvalbumins and tropomin C, the myosin P-light chain and calmodulin (Collins, 1976; Perry, 1979). Parvalbumins are able to inhibit myofibrillar ATPase activity by binding Ca^{2+} (Pechere et al., 1977), and the Ca^{2+} may be exchanged and accumulated by S.R. vesicles (Gerday and Gillis, 1976). It has therefore been postulated that the function of parvalbumins is to induce rapid relaxation in fast fibres (Gerday and Gillis, 1976; Pechere et al., 1977). Ca^{2+} release by the S.R. will cause only a transient activation of the contractile apparatus through troponin C, as Ca^{2+} is quickly sequestered by the parvalbumins and subsequently accumulated by the S.R. In conjunction with a high rate of rise of tension, this would result in a very short activation/relaxation cycle, allowing a fish to achieve very high tailbeat frequencies during burst swimming.

Fibre Recruitment During Swimming

The available evidence suggests that the pattern of fibre recruitment at different swimming speeds parallels the type of fast muscle innervation. Electromyographical studies during swimming have been carried out on two fish having focally innervated fast fibres, the dogfish (Bone, 1966) and the Pacific herring, Clupea herangus (Bone et al., 1978). In both fish, only the slow fibres are active at low sustainable swimming speeds, and at higher speeds, where fast fibres must be recruited, the fish fatigue in only 1-2 minutes.

The division of labour is not so well defined in fish with polyneuronally innervated fast fibres. Fibre recruitment has been studied

in skipjack tuna (Rayner and Keenan, 1967; Brill and Dizon, 1979), rainbow trout (Hudson, 1973), carp (Johnston et al., 1977), striped bass, Morone saxatilis and bluefish, Pomatomus sultatnix (Freadman, 1979), brook trout (Johnston and Moon, 1980b) and coalfish, Pollachius virens (Johnston and Moon, 1980a). At low speeds, only the slow fibres are active, but as swimming speed increases, a threshold is reached where fast fibres are recruited. This speed ranges from 0.8-0.9 Ls^{-1} in coalfish to 4.5 Ls^{-1} in bluefish. These speeds are sustainable for many hours or days (e.g., Johnston and Moon, 1980a,b). Only in the carp has the recruitment of the intermediate "pink" fibres been studied (Johnston et al., 1977). As swimming speed increased, the pink fibres were recruited before the fast. Although it has not been investigated thoroughly, it appears that preferential recruitment on the basis of depth within the myotome may occur (Johnston and Moon, 1980a). At intermediate swimming speeds, superficial fast fibres appear to be recruited before those next to the vertebral column.

It is interesting to note that the e.m.g. activity from the fast muscle at slow speeds in carp and coalfish (Johnston et al., 1977; Johnston and Moon, 1980a) is similar to that from slow muscle. Higher amplitude spike-like potentials are seen during high speed and burst swimming. The occurrence of both junction potentials and action potentials in fast muscle (Hudson, 1969) has been referred to above. Are local, graded contractions responsible for fast muscle activity at low swimming speeds? At this stage, we can only speculate on the answer.

Metabolism

The metabolic cost of swimming increases approximately exponentially with speed, due to the hydrodynamic constraints imposed on the fish. The metabolic "design" has evolved to meet these special demands. Although the energy cost of locomotion is in fact lower in fish than in

mammals, fish have only 1-10% the aerobic scope of mammals (Brett, 1972), showing a high dependence on anaerobic metabolism.

In vitro determinations (under optimum conditions) of the activities of key enzymes involved in energy metabolism help to define the relative importance of particular pathways, and a number of such studies have been carried out (e.g., Crabtree and Newsholme, 1972; Johnston et al., 1977; Guppy et al., 1979; Johnston and Moon, 1980a). In general, phosphorylase, phosphofructokinase and other glycolytic enzyme activities are 2-3 times greater in fast muscle than in slow, but exceptions do occur (e.g. in Anguilla, Boström and Johansson, 1972). Markers for oxidative pathways, e.g. citrate synthetase, usually have higher activities in slow muscle (Johnston and Moon, 1980a). ATP turnover has been shown to be three times greater in fast muscle, and the ATP "regenerating" enzymes, creatine kinase and adenylate kinase parallel these differences. Note, however, that relative anaerobic capacities can be modified by external factors such as temperature acclimation (Hazel and Prosser, 1974), endurance exercise training (Johnston and Moon, 1980a), development (Boström and Johansson, 1972) and starvation (Moon and Johnston, 1980).

Metabolism during swimming is, as yet, poorly understood, since proper control of the activity of fish before and after the exercise period has not always been achieved. Some general conclusions can, however, be drawn. Oxygen uptake can increase 10-15 times between rest and maximum activity (Bennet, 1978), and it seems likely that the slow muscle, with its active aerobic metabolism, receives a significant proportion of the cardiac output during steady swimming. Lipids are the major slow muscle fuel during this kind of activity (Bilinski, 1974). In elasmobranchs, the enzymes of ketone body oxidation have high activities, e.g. 3-hydroxybutyrate dehydrogenase (Zammit and Newsholme, 1979). In the teleost, however, the activities of the enzymes involved

in fatty acid oxidation are high, e.g. triacyl glycerol lipase and high concentrations of triacyl glycerol and non-esterified fatty acids are found in the plasma (Zammit and Newsholme, 1979). Ketone bodies therefore appear to be the most important lipid fuel in elasmobranchs, with fatty acids of greater importance in teleosts. Fast muscles appear to utilise lipids to a much lesser extent, even in teleosts, during steady swimming (Jonas and Bilinski, 1964; Bilinski, 1974; Crabtree and Newsholme, 1972).

Glycogen is also used, in both fibre types, during steady swimming (Pritchard et al., 1971; Johnston and Goldspink, 1973a,b). In carp, swimming at 3 Ls^{-1}, the rate of glycogen utilisation is 2-3 times greater in slow muscle than in fast (Johnston and Goldspink, 1973c). However, the slow muscle constitutes only 7% of the myotomal mass, and 80-85% of the glycogen is therefore utilised by the fast muscle. The importance of aerobic glycolysis varies considerably from species to species (Newsholme et al., 1978; Johnston and Moon, 1980a; Johnston, 1977; Guppy et al., 1979; Walesby and Johnston, 1980). In the fast fibres of trout, high hexokinase activity (Johnston and Moon, 1980b), high mitochondrial number (Nag, 1972) and high citrate synthetase and cytochrome oxidase activities (Johnston and Moon, 1980b) suggest that the capacity for aerobic glycolysis may be sufficient to support sustained activity. If trout are trained for several weeks prior to experimentation to prevent stress, no net accumulation of lactate is detected at speeds of up to 4 Ls^{-1} (Johnston and Moon, 1980a). The situation may be different for other species. For example, Smit et al. (1972) calculated, on the basis of O_2 consumption measurements and efficiency of swimming, that in goldfish (Carassius auratus), 80% of their energy requirements came from anaerobic pathways. Much of the lactate produced is oxidised to pyruvate (Bilinski, 1974). The gills, liver, red muscle and kidneys can all oxidase lactate at high rates in vitro (Bilinski and Jonas,

1972), but the relative importance of these sites is still in debate (see Johnston, 1981b).

Burst swimming is accompanied by the rapid activation of glycolysis, which in trout depletes muscle glycogen by 50% in 15 s (Stevens and Black, 1966), almost all of which is converted to lactate (Wardle, 1978). Recovery to normal lactate levels usually takes 30-60 min. in slow muscle (Johnston and Goldspink, 1973a), but may take up to 18 h. in white muscle (Black et al., 1961).

Table 1:1: Fractional volumes occupied by various cellular components in fish fast and slow myotomal muscle. Refs. 1 - Patterson and Goldspink, 1972; 2 - Bone, 1978a; 3 - Walesby and Johnston, 1980; 4 - Kryvi et al., 1980; 5 - Nag, 1972; 6 - Kryvi, 1977; 7 - Kryvi, 1978; 8 - Johnston and Maitland, 1980; 9 - Totland et al., 1981; 10 - Johnston and Bernard, in preparation; 11 - Johnston, 1981a in press.

Parameter % fractional volume	Fast	Slow
Mitochondrial (Refs. 1-4)	0.5-9	25-35
Sarcoplasmic reticulum (Refs. 1, 5-7)	6-14	4.6-5.1
T-system (Refs. 1, 5-7)	0.4-0.9	0.1-0.6
Myofibrils (Ref. 8)	67-83	43-60
Capillaries (% fibre surface vascularised) (Refs. 9-11)	3.6-7.0	14.7-51

CHAPTER 2

QUANTITATIVE HISTOCHEMICAL STUDIES OF THE PERIPHERAL INNERVATION OF COD (GADUS MORHUA) FAST MYOTOMAL MUSCLE FIBRES

INTRODUCTION

In elasmobranchs, chondrosteans, dipnoans and some primitive teleosts, fast fibres receive a single motor end plate consisting of numerous basket-like terminations (Barets, 1961; Bone, 1964). Electromyographical studies in these fish have shown that fast muscles are reserved for short bursts of high speed swimming (Bone, 1966; Bone et al, 1978). Apparently, the small percentage of highly aerobic slow fibres (5-15%) is sufficient to provide all the power required for sustained activity. Fast muscles in elasmobranchs are poorly vascularised, and almost entirely dependent on anaerobic glycogenolysis for their energy supply (Kryvi, 1977; Bone, 1978).

Advanced teleosts have an unusual pattern of fast muscle innervation. Instead of a single motor end plate each fibre receives a dense network of innervation with as many as 23 terminations per fibre (Barets, 1961; Bone, 1964; 1970; 1975). Electrophysiological investigations have found evidence for extensive polyneuronal innervation with fibres receiving axons derived from more than one spinal root (Takeuchi, 1959; Hagiwara and Takahushi, 1967; Hudson, 1969).

The acquisition of this type of innervation is a late evolutionary development among the vertebrates and has enabled fast muscles to be recruited over a wide range of swimming speeds (see Bone, 1978; Johnston, 1980b). For example, the threshold speeds at which e.m.g.s can be recorded from fast muscles is 1.4 body lengths/s in rainbow trout (Hudson, 1973), 0.5 body lengths/s in carp (Bone et al., 1978)

and 0.8-1.9 body lengths/s in saithe (Johnston and Moon, 1980a). These are all swimming speeds that can be maintained indefinitely. In addition to a highly developed glycogenolytic potential fast muscles in these species also have a significant aerobic capacity which may be 15-20% that of slow fibres (Johnston and Moon, 1981). Fast myotomal muscle consists of fibres with a wide range of sizes (Green, 1913; Boddeke et al., 1959). Recent quantitative ultrastructural analyses (Johnston and Moon, 1981) have provided evidence for a significant hetereogeneity in the aerobic capacity of fast muscle in some species. In general, small fast fibres have higher glycogen and mitochondrial contents than large fibres (Boddeke et al., 1959).

Hudson (1969) found that stimulation of spinal nerves produced two distinct electrical responses from the abdominal myotomes of the short-horned sculpin (Myoxocephalus scorpius) namely local junction potentials leading to a graded contraction, or propagated action potentials resulting in a fast twitch. One possibility discussed by Hudson (1973) is that the range of fibre size found within the fast muscle may reflect the presence of fibres with different membrane properties and hence mechanical responses and functions in swimming.

There has only been one previous quantitative study on the myotomal innervation of a teleost fish (Hudson, 1969). In the present study the peripheral innervation of cod (Gadus morhua) fast fibres has been investigated after staining the endplates and preterminal axons for cholinesterase activity.

MATERIALS AND METHODS

Fish

Cod (Gadus morhua) were netted in the Firth of Forth by commercial fishermen during September to October 1980 and kept in the laboratory

in recirculated filtered sea water at 10±1°C. Fish were used within one week of capture.

Tissue sampling and histochemistry

Four fish were used in the study, all of comparable size and weight (length = 33 cm/370g; 34/385; 33/333; 34/360). Counting from the head, all samples were taken from myotomes 18-20, and within 1 cm either side of the lateral line (see Figure 2:1). After removal of the red and intermediate fibres, superficial and deep samples of the fast muscle were taken as illustrated in Figure 1:1. Bundles of fibres were excised, complete with myoseptal attachments and pinned out on small cork slabs. Preterminal axons and neuromuscular junctions were stained using the acetylthiocholine technique of Naik (1963). After fixing in 10% formalin (in 0.1M, acetate buffer pH 5.2) for two hours at 10°C, fibres were washed for one hour in distilled water. Incubation in the acetylthiocholine solution was carried out at 37°C for 6-8 hours. Bundles incubated with Eserine Sulphate were used as controls. After staining in Ammonium Sulphide and washing and clearing in glycerol, small fibre bundles of 1-6 fibres, and larger bundles of 10-50 fibres (up to four fibres deep), were mounted in glycerol. Measurements were taken directly from the slides under an optical microscope and from photographs. Statistical analyses were carried out using a students t-test.

RESULTS

The number of end plates per fibre were counted for 50 deep and 50 superficial fast fibres from each fish, and the results pooled (Figures 2:2 and 2:3). The mean number of end plates was 17.88 ± 2.13 (mean ± 1 S.D.) for the superficial and 14.79 ± 2.48 for the deep fibres, $P << 0.001$. End plates are distributed uniformally along the fibres.

No correlation was found between the number of end plates per fibre and fibre diameter (Figure 2:4).

In the more intact preparations, the terminal branches of an axon could be traced to their end plates on the fibres. Figure 2:5 shows the distribution of fibre diameters innervated by 20 typical terminal axons. It is clear that a given axon innervates fibres with a wide range of diameters (see also Figure 2:2e).

DISCUSSION

One of the first detailed descriptions of teleost myotomal muscle innervation was made by Barets (1961). In describing tench fast muscle, he stated that branches of the spinal nerves running in the myosepta fan out to form a diffuse network over the fibres, each fibre receiving many terminations. Subsequent studies by Bone (1964) and Hudson (1969) found that each spinal nerve runs along a myoseptum giving off branches to the adjacent myotomes anteriorly and posteriorly. Branches occasionally cross myosepta to joint the network of subadjacent myotomes. Small branches, containing a variable number of axons, run for short distances between the fibres. Axons from one bundle do not all terminate on the same muscle fibre. The situation is obviously extremely complex. Qualitative studies on the pattern of innervation and the structure of the neuromuscular end plates have been done by a number of workers (Barets, 1961; Bone, 1964; Hudson, 1969; Nishihara, 1967), but few quantitative studies have been attempted. Hudson (1969) working on Myoxocephalus scorpius with histochemical and electrophysiological techniques concluded that each fibre receives innervation from up to 5 axons (usually 2-3) from each of four spinal nerves. The number of terminations per fibre varied from 8-22, with a mean of 14.5 (from 52 fibres). He concludes that each end plate is derived from a separate

axon, and estimates 12 motor units per abdominal myotome, all of considerable size. The number of end plates per fibre in cod (Gadus morhua) is similar to that of Myoxocephalus scorpius (Hudson, 1969) (14.8 deep, 17.9 superficial, range 9-23). Hudson describes the end plates as "multiple, very variable in extent and form, and localised in regions along each fibre" (Hudson, 1967). Axons exhibit preterminal and terminal branching before synapsing on a particular fibre in sequential, bead-like expansions, extending over several hundred µm (Hudson, 1967; 1969). In counting end plates, each region was described as one termination. An essentially similar structure was seen in the present study (Figure 2:2). An axon, in a small number of cases, would, after branching, terminate as two discrete end plates on opposite sides of the fibre. The distance between two such terminations could be as great as that between two end plates from different axons. For this reason, the fibres were considered to be multiterminally innervated. Although Hudson suggests that the white muscle of Myoxocephalus is only polyneuronally innervated he points out that his studies cannot rule out a small degree of multiterminal innervation such as described in the present study.

Johnston (1980a) has shown that tetanic fusion frequencies for cod white myotomal muscle are typically 40-50 Hz. Maximal tension is not reached until stimulation exceeds 200 Hz. The rate of tension development is also highly frequency dependent, again reaching maximal values above 200 Hz. Alexander (1969) has shown that teleost fast muscle undergoes very little shortening during swimming (approximately 2-3% of Lo). It is therefore possible that the rate of tension development is determined as much by the muscle membrane properties as by its intrinsic maximum contraction velocity. If the fast fibre mass is made up of a number of functionally distinct populations, then this may be reflected in the peripheral innervation pattern. However, as shown in

Figure 2:4, there is no correlation between fibre diameter and the number of end plates observed.

Assuming that the degree of multiterminal innervation is constant throughout the myotome then it is likely that each fibre will receive, on average, terminations from the same number of axons, irrespective of size. From Figure 2:5 it is seen that a given axon will terminate on fibres of greatly varying size. Thus, stimulation of a small fibre through such an axon will also stimulate the larger fibres. Since the number of endings, and hence the number of axons per fibre, is independent of size, then it would seem unlikely that there could be selective activation of a particular fibre size population unless their membrane properties were different. If the smaller fibre, for example, had a lower threshold potential for the generation of action potentials, then at a given rate of stimulation they may be fully activated when the larger fibres are giving only the local contractions associated with junction potentials.

An interesting finding was that the mean number of end plates on the deep fibres was significantly lower than on the superficial fibres. Again, assuming a constant degree of multiterminal innervation, the deep fibres are innervated on average by fewer axons. Since such high frequencies of stimulation are required for activation it is possible that the superficial fibres, with their greater number of axons, will be recruited first as nervous stimulation increases. For a given level of stimulation the deep fibres will always be less active.

Electromyographical studies on the carp have shown a sequential recruitment of fibre types in the order red, pink, white as swimming speed increases (Johnston et al., 1977).

Johnston and Moon (1980b) working on saithe (Pollachius virens) noted that the threshold speeds for recording e.m.g.s from superficial fast fibres (0.8 body lengths/s) was lower than that for deep fast

muscle (1.9 body lengths/s). There is thus some evidence for recruitment according to position, within the fast muscle. These observations in themselves do not seem compatible with recruitment on the basis of fibre size.

Figure 2:1: Transverse section through the fish at myotome 18 (counting from the first myotome behind the operculum). R = red muscle, W = white muscle. DW, SW = sampling areas for deep and superficial white muscle respectively.

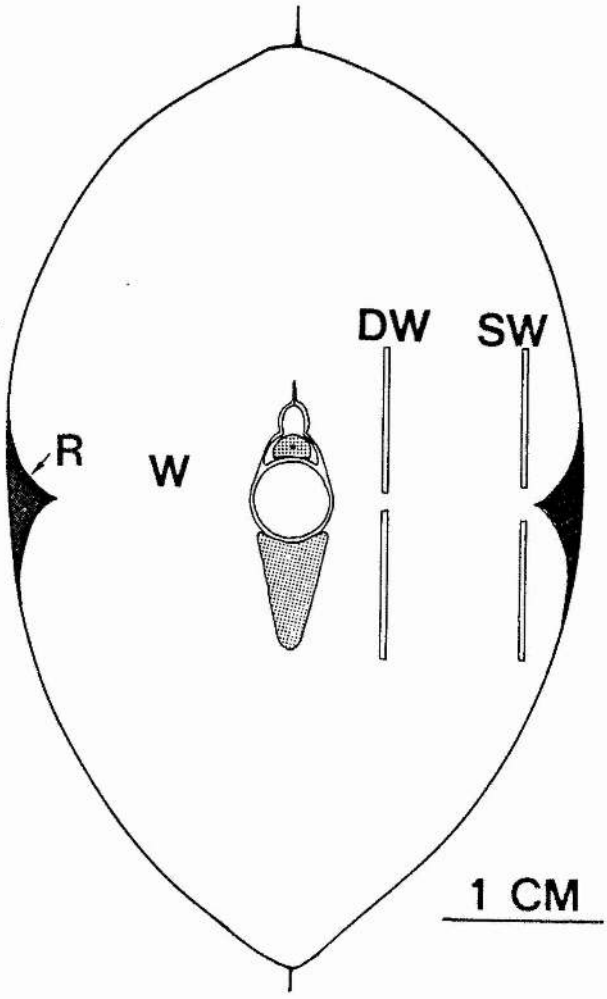
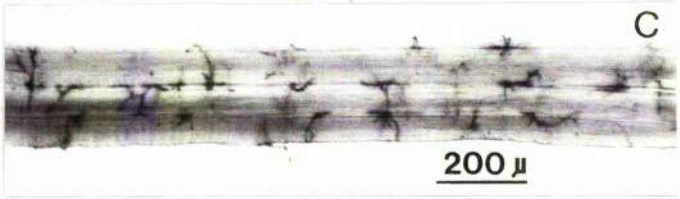
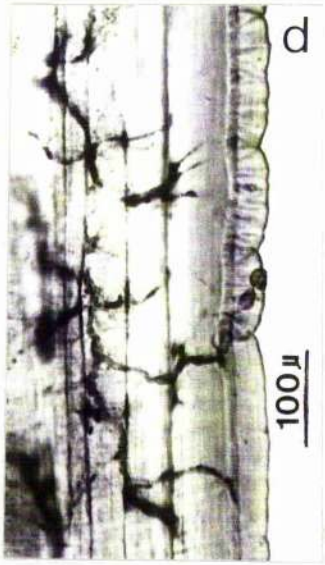

DW
SW
R
W
1 CM

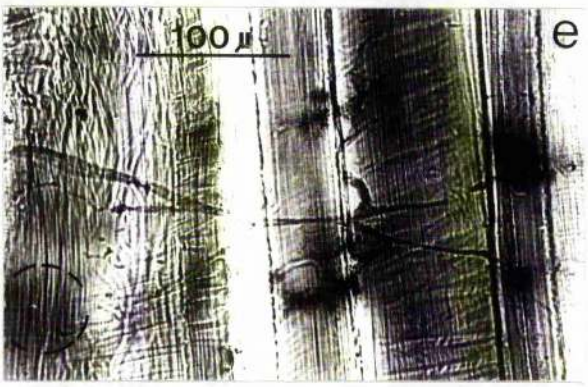
Figure 2:2: a, b, c low power photomicrographs showing the general distribution of end plates. The variety of form is illustrated in d-g. The wide range of fibre diameters innervated by one axon is shown in e. The dotted line indicates an end plate, deeper in the section, terminating on the large diameter fibre on the left. Multiterminal innervation is demonstrated in d and e.

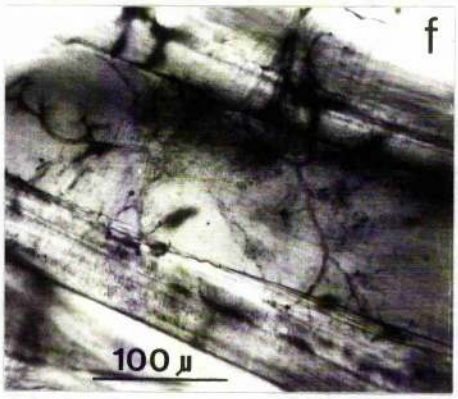

a
1mm


b
200µ


c
200µ


d
100µ


100µ
e


f
100µ

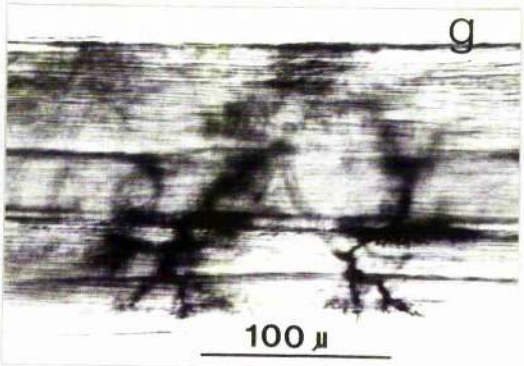

g
100µ

Figure 2:3: Histogram of the number of fibres against the number of end plates per fibre. Superficial fibres, mean = 17.88 ± 2.13 (mean ± 1 S.D.), deep fibres, mean = 14.79 ± 2.48 (mean ± 1 S.D.).

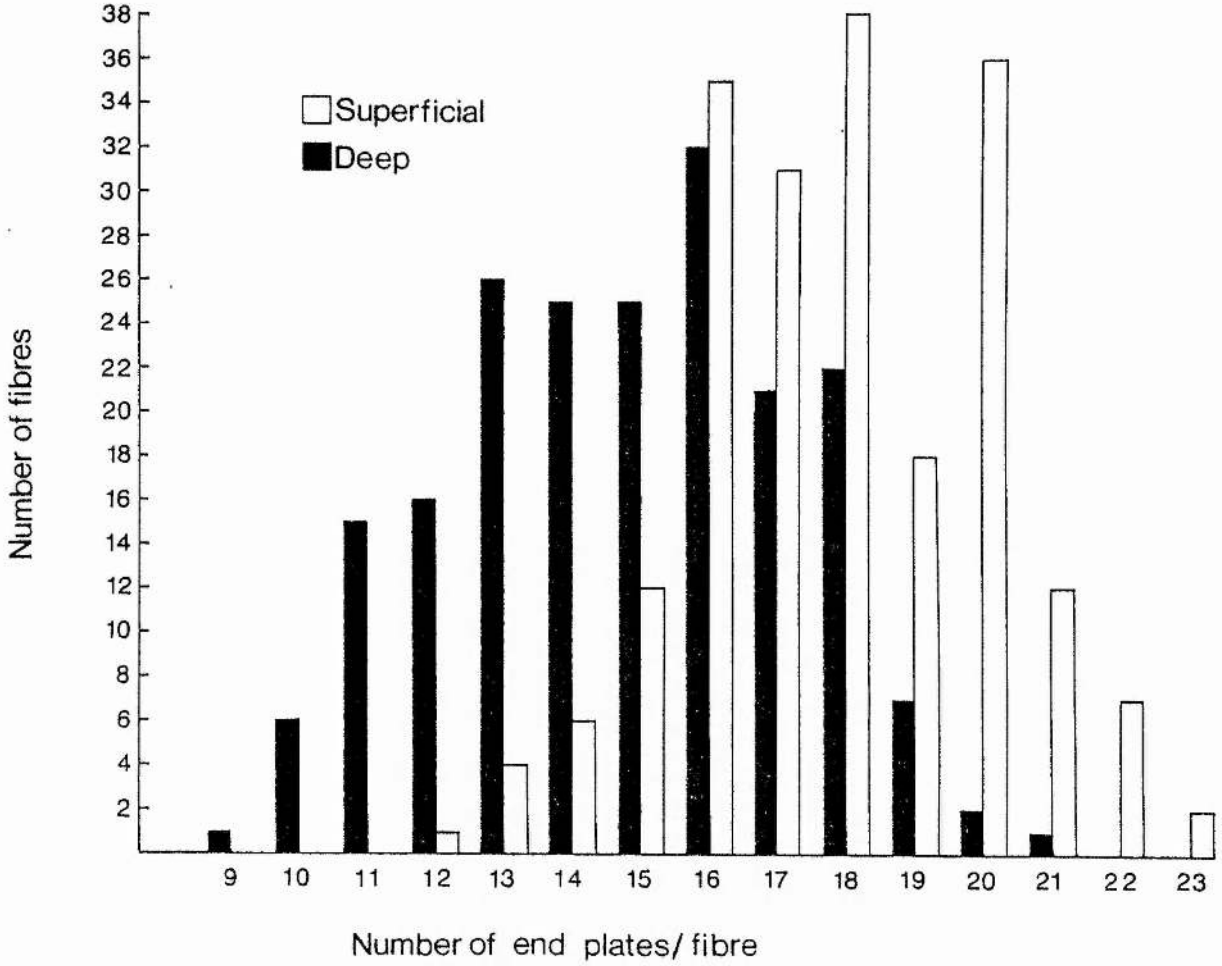

Superficial
Deep
Number of fibres
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
Number of end plates/ fibre

Figure 2:4: Number of end plates per fibre plotted against fibre diameter.

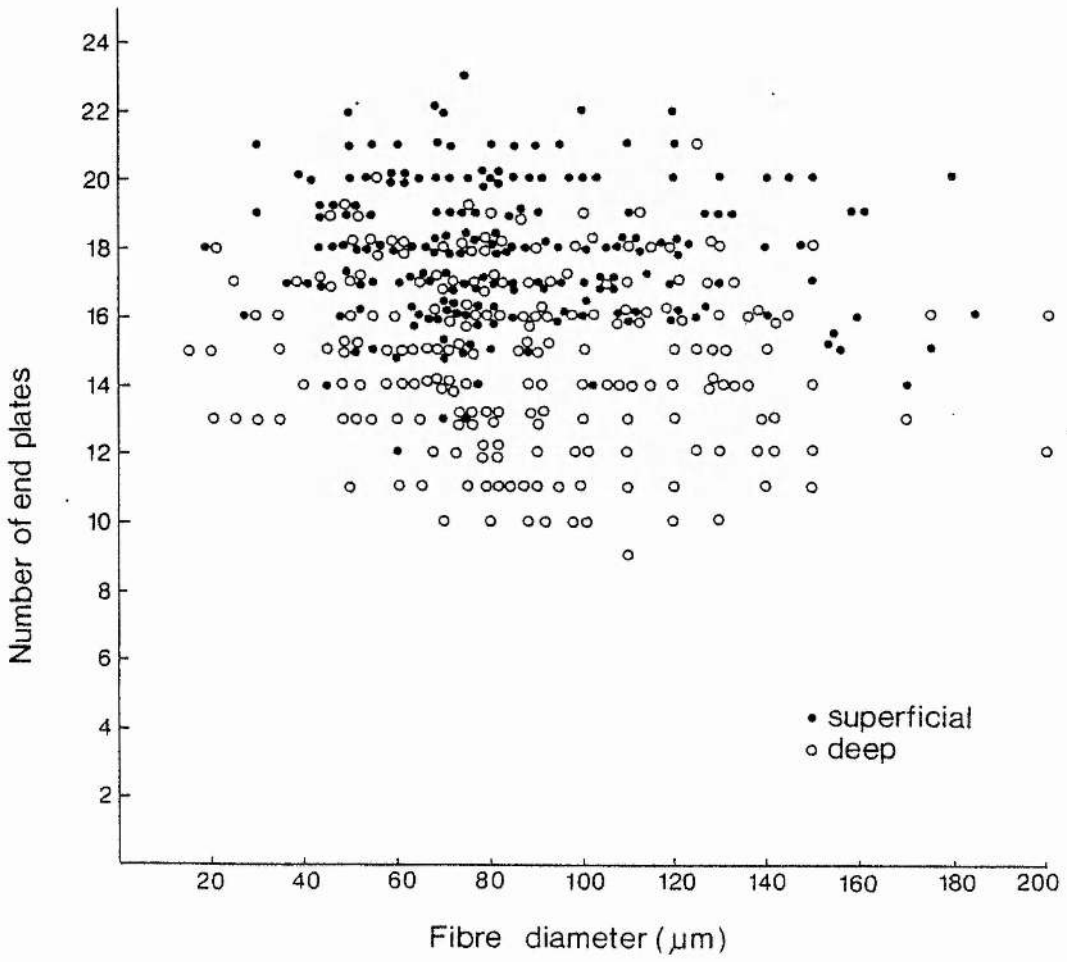
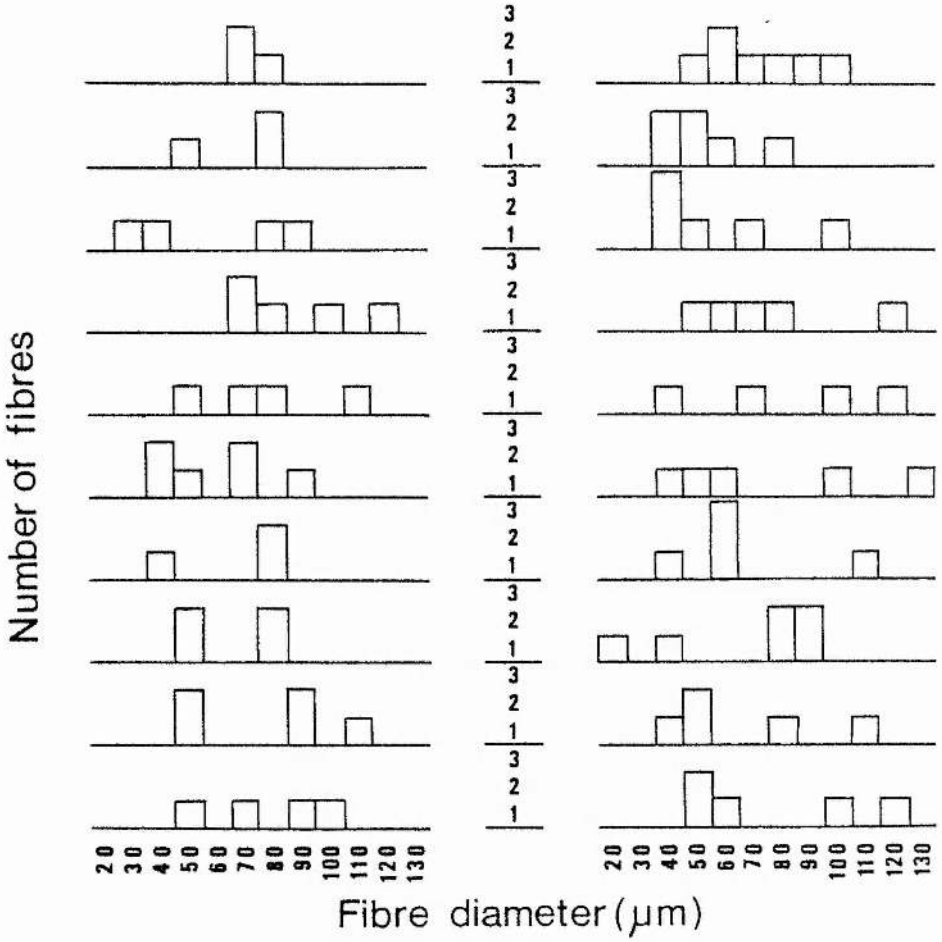

Number of end plates
24
22
20
18
16
14
12
10
8
6
4
2
20
40
60
80
100
120
140
160
180
200
Fibre diameter (μm)
• superficial
o deep

Figure 2:5: Distribution of fibre diameters innervated by 20 typical terminal axons.


Number of fibres
3
2
1
20
30
40
50
60
70
80
90
100
110
120
130
Fibre diameter (μm)

CHAPTER 3

THE MECHANICAL PROPERTIES OF ACTOMYOSIN THREADS

INTRODUCTION

Actomyosin threads provide a potentially useful tool for investigating the mechanisms of force generation in contractile systems. Using intact and skinned fibre preparations the mechanical properties of muscle can be studied directly, but a suitable preparation can not always be obtained from the system under study. For example, the complex arrangement of fibres within the myotomes of fish musculature exclude all but the largest fish from single fibre studies. An intact single fibre preparation requires strong mechanical attachment to the apparatus, which does not damage the sarcolemma. Long tendons are normally left on a single fibre after dissection, and these can be firmly held in the apparatus, close to the fibre, giving a low compliance attachment without risk of damage to the cell membrane. Fish fibres are inserted into thin, sheet-like myosepta. Fibres in adjacent myotomes insert into the other face of this sheet. No firm mechanical ground can be made to this delicate septum without damage to the muscle fibre itself, except in very large fish (> 50 cm).

Much can be learnt from experiments involving chemical modification of the contractile proteins. For example, Wagner and Weeds (1977) cross hydridized fast and slow muscle myosin light chains from rabbit muscle with fast muscle S_1 heads. They were able to reversibly confer fast or slow muscle ATPase activities on the myosin heads - an elegant demonstration of the role of the myosin light chains in determining cross bridge cycling rate. The degree to which skinned fibres can be

chemically modified is limited, and modification of proteins for experiments in solution uncouples the biochemical and mechanical events. Actomyosin threads may fill the gap, providing a force generating model in which protein composition may be changed, and the proteins themselves chemically modified in isolation.

Actomyosin threads were first made by Boehm and Weber in 1932 and, in 1939, Engelhardt and Ljubimova demonstrated the plasticising effect of ATP hydrolysis in threads. ATP hydrolysis was also shown to be associated with shrinkage (Szent-Györgyi, 1942). However, Portzehl and Weber (1950; 1954) were the first to attempt to measure force generation in the presence of ATP. They precipitated their threads by extrusion of a solution of actomyosin through a syringe, into a solution of low ionic strength. Threads were then dried to increase the protein concentration to 60-100 mg ml^{-1}, and isometric tensions of 200-300 g cm^{-2} were measured. Hayashi (1952) prepared threads by compressing monolayers of natural actomyosin in a Langmuir trough. He demonstrated that the degree of shortening in the presence of ATP was dependent upon the load. Shortening velocity was initially rapid, but slowed throughout contraction, which was still incomplete after 40 minutes. In all of the above experiments, tensions were poorly correlated with the protein content of the threads, which could vary greatly from preparation to preparation. For this reason, and because of the difficulty of accurately measuring such small tensions, actomyosin threads received very little attention for the next 20 years. D'Haese and Komnick (1972) studied the ultrastructure of extruded threads under the electron microscope, and concluded that contraction is by a sliding filament mechanism, similar to that which is thought to operate in muscle. Their work will be discussed more fully at the end of the chapter.

Advances in electronics encouraged further work. Crooks and Cooke (1977) designed and built a sensitive tensiometer which could

reproducibly measure the small tensions (1-5 mg) produced by isometrically contracting threads, and their isotonic contraction velocities under different loads. During recent years, the contractile proteins of muscle have been isolated and purified, and the techniques have become standard in many laboratories. The potential uses of actomyosin threads were therefore greatly increased. Using the extrusion method, protein content could be kept constant, and threads of a given, uniform diameter could be produced. Maximal isometric tensions were very much lower (10 g cm^{-2}) than those obtained from intact and skinned fibre preparations. Unloaded contraction velocities were around 10^{-2} L s^{-1} 2-3 orders of magnitude lower than those of intact muscle fibres. Threads of purified actin and myosin did not require Ca^{2+} for contraction in the presence of ATP. The addition of the regulatory proteins conferred Ca^{2+} sensitivity or the threads, and tension showed a dependence on free [Ca^{2+}] similar to that of muscle. However, removal of Ca^{2+} ions by the addition of EGTA was only partially successful in relaxing the threads. As with intact fibres (Gulati, 1976), isometric tension and V_{max} decrease as the temperature is lowered. P_0 and V_{max} were only slightly affected by changes in the ionic strength. In intact fibres, increasing [KCl] decreases P_0, but does not greatly affect V_{max} (Gordon and Godt, 1970; Lännergren and Noth, 1973). Similar results were found for skinned fibres (Gordon et al., 1973; Thames et al., 1974).

Using a chemically modified myosin, Cooke and Franks (1978) investigated the possibility of cooperativity between the two S_1 heads. Digestion with papain and subsequent purification produces single headed myosin which is 90-95% pure. When incorporated into actomyosin threads, the contraction velocities of threads with single headed myosin did not differ from those with normal double headed myosin. The tension produced per S_1 head was also similar in both types of thread.

It was concluded that the absence of one head did not impair the force generation or motion of the other.

Non-muscle actomyosins have also been studied. The great potential of threads is illustrated here, in the study of systems from which one cannot obtain conventional mechanical preparations. Lebowitz and Cooke (1978) purified actomyosin from human blood platelets to make threads. Isometric tensions and contraction velocities were similar to those of skeletal muscle threads. Maximum tension was found to be directly proportional to the level of phosphorylation of the 20,000-dalton myosin light chain. They suggest that phosphorylation of platelet myosin "switches on" its ability to interact with actin. Matsumura et al. (1980) studied the properties of actomyosin threads from a slime mould, Physarum polycephalum, and found tension to be reversibly dependent upon ATP concentration between 0 and 20 μM. Their properties were essentially similar to skeletal muscle actomyosin threads, but tension development was somewhat slower, full tension not being reached for up to 8 minutes.

APPARATUS

Principals of Operation

The apparatus is designed to work in both isometric and isotonic modes. Tensions measured are in the range 1-5 mg. Figures 3:1 and 3:2 show details of the lever, photodiode assembly, and a block diagram of the associated electronics. A lightweight aluminium lever, pivoted near the centre has a flag attached at one end, which moves in the path of the light beam falling on the photodiode.

In isometric, contraction of an actomyosin thread attached between the two hooks causes a deflection of the flag. The increased photodiode current is fed back into a lightweight coil, suspended from

the lever, in the field of a permanent magnet. This produces a restoring force, holding the thread at L_0. Isometric tension is proportional to the voltage across the coil. Switching the relay to the isotonic mode allows the thread to contract against a load determined by the preset current in the coil. Photodiode output is proportional to displacement. Therefore, displacement against time is contraction velocity. Sensitivity in isometric = 135 mV mg^{-1}, in isotonic 70 μm/100 mV. Compliance in isometric is < 0.1 μm mg^{-1}, and drift < 0.02 mg hr^{-1}. The switch to isotonic takes < 10 ms.

Development and Detailed Operation

The transducer is a development of that described by Crooks and Cooke (1977). The circuit was modified to give the extra facilities of a large d.c. offset voltage, and a variable dynamic range. Crooks and Cooke used the movement from a Cahn electrobalance in their apparatus. We tried a number of small moving coil movements without success. The negative feedback system in use requires some damping for stability. The restoring force produced by displacement causes overshoot of the lever. This in turn produces a restoring force in the opposite direction. If left, positive feedback will develop, and the lever will oscillate. The frequency of oscillation is inversely proportional to the time constant of the electronically applied damping. If this time constant becomes very large, then during a contraction the rate of rise of voltage across the coil may be determined by the capacitance of the damping circuit, and not the rate of tension development, which may have a smaller time constant. A lever system is therefore needed in which the damping sufficient to prevent positive feedback oscillations has a time constant much smaller than those expected of tension development curves. Stability in the isometric mode is determined by the dimensions and mass of the lever, and the relative positions of the

fulcrum and coil. The mass of the beam must be kept as small as possible, a large moment of inertia will decrease the apparent contraction velocity. The lever must, however, have a low compliance. To meet all of these criteria, a purpose built lever was made (Figure 3:1). It is constructed from two thin walled aluminium tubes 12 cm long, mounted one on top of the other, to increase stiffness in the direction of loading. A similar aluminium tube (5.5 cm long) projects downwards at one end, and a small platinum hook is attached to it. The lever is pivoted on diamond watch bearings 5.8 cm from the hook. At the other end an aluminium foil flag projects 1 cm into the photodiode assembly. A small aluminium foil former, suspended from the lever by foil tabs, bears a hand wound coil of fine copper wire. The coil moves in a circular hole in the top of a large permanent magnet. The ends of the coil are soldered to contacts on the magnet. The photodiode and an infrared L.E.D. are held in a metal housing, adjustable in the vertical plane. The whole assembly is built into a draught proof casing, designed to allow easy access for attachment of a thread to the hooks. The lower, fixed hook, and the lever housing, can be moved independently on micromanipulators.

The subunits of the circuit are shown in Figure 3:2 and the circuit diagram in Figure 3:3. The photodiode (PIN SPOT 2D, U.D.T. Santa Monica, California) has two light sensitive panels. The upper is constantly illuminated, serving as a reference against fluctuations in light intensity emitted by the L.E.D. The lower monitors the position of the flag. The outputs from the two cells are fed into the X_{sj}, Z_{sj} voltage inputs of an Analog Devices 434B analogue divider. This is a one quadrant divider, with an accuracy of > 0.25% over a 100:1 range of denominators.

$$\text{Output } e_0 = \frac{10}{V_{Ref}} V_Y \frac{I_Z}{I_X} .$$

The V_Y pin is a 9 V reference voltage. When connected to the V_{Ref} pin, the equation becomes

$$e_0 = 10 \frac{I_Z}{I_X} .$$

Z_{sj} is connected to the upper cell, X_{sj} to the lower cell.

With the relay set in the isometric position, the output of the divider is fed into the positive input (1) of I.A.1, an Analogue Devices 521JD instrumentation amplifier. It has an accurately programmable input/output gain of 0.1-1000, low gain drift, and a high input impedance (3×10^9 Ω). The gain is set to 1.A 3V reference voltage from the stabilised power supply is fed to the negative input (3). The output is amplified five-fold by a 741 operational amplifier circuit, and passed to the coil. Damping is provided by a series of capacitors linked to the 741 circuit by a double ganged switch, through the relay. At maximum damping, the time constant is approximately 2 s. Switching to isotonic removes the damping from the circuit.

In isotonic, the divider output is fed to I.A.2, a second instrumentation amplifier, with a variable gain of 0.1, 1, or 5, and the output fed into a second 741 operational amplifier, with a gain of 1. A large d.c. voltage can be applied to the circuit at this point as an offset. The output of the 741 is connected to the oscilloscope/chart recorder. An isotonic load can be applied to the coil through the isometric amplifier. A 50 K, 10 turn potentiometer in a voltage divider circuit is used to set the load, and its value is measured as a voltage, by a voltmeter connected across a 5 K preset resistor in series with the coil. Adjustment of the preset resistor allows the dynamic range of the apparatus to be altered.

The relay switches from isometric to isotonic in < 10 ms. Switching in the opposite direction takes over 2 s, to avoid damage to

the threads by rapid stretching.

METHODS

Materials

Dogfish, Scyliorhinus canicula, were netted in the English Channel near Plymouth. They were kept in filtered recycled seawater at 10°C ± 1°C in large tanks at the Marine Biological Association Laboratories, Citadel Hill, Plymouth, where the work was undertaken. Fish were used within one week of capture.

Rabbits were obtained from a local supplier.

Dissections

Fish were killed by decapitation, and the red and white myotomal muscle quickly dissected. Rabbits were killed by a blow to the head. The heart was removed and a 2-3 g sample of the longissimus dorsi muscle (predominantly fast glycolytic fibres) dissected. The heart was rinsed 2-3 times in cold 0.1 M KCl, 5 mM imidazole pH 7.0. All muscles were stored on ice during dissection.

Preparation of Natural Actomyosin

The method is essentially that of Lehman and St.Györgyi (1975).

The muscle tissue is finely minced, and washed three times in four volumes (to one volume of muscle) of 0.1 M KCl 5 mM imidazole pH 7.0, by low speed centrifugation at 2°C (M.S.E. Coolspin, 2500 r.p.m.) and resuspension in fresh solution. Four volumes of cold 0.6 M KCl, 5 mM imidazole pH 7.0, are added to the pellet, and gently stirred for 2 hours at 4°C. The insoluble material is removed by centrifugation at 2°C (30,000 g, 20 min.). The supernatent is filtered through glass wool, and slowly diluted, with stirring, to 0.1 M KCl by the addition of cold distilled water to precipitate the natural actomyosin. The

actomyosin is pelleted by high speed centrifugation (43,000 g for 40 min.). The pellet is solubilised by the addition of 2.5 M KCl to give a final concentration of 0.6 M KCl. The actomyosin is now ready for thread extrusion.

All solutions contain 0.1 mM DTT (or DTE or 2 mercaptoethanol).

Preparation of Myosin

The method is that of Barany and Close (1971). The muscle is finely chopped, and homogenised in 10 mM KCl at 0°C for three periods of 20 s, with 20 s pauses to prevent overheating. The solution is then made up to 0.3 M KCl, 0.15 M potassium phosphate buffer, 10 mM ATP and 1 mM cysteine pH 6.6. 25 volumes of this solution are added for every g. of fresh muscle. The extract is gently stirred for 20 minutes at 4°C, and the insoluble material removed by centrifugation at 30,000 for 20 minutes. The supernatent is dialysed at 4°C for 36 hours against four changes of 150 volumes of a solution containing 10 mM KCl, 1 mM imidazole, 0.5 mM cysteine pH 7.0. The precipitated myosin is pelleted by centrifugation at 43,000 g - 40 min.) and redissolved in 2.4 M KCl to a final concentration of 0.6 M KCl.

Preparation of Actin

The method was based on that of Spudich and Watts (1971).

Stock solutions:

(A) 2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$ 0.5 mM 2 mercaptoethanol pH 8.0;

(B) 0.15 M $KHPO_4$, pH 6.5, 0.3 M KCl, 0.5 mM 2 mercaptoethanol;

(C) 0.4% $NaHCO_3$;

(D) 50 mM $NaHCO_3$, 50 mM Na_2CO_3;

(E) 0.5 mM $CaCl_2$, 0.5 mM 2 mercaptoethanol.

Preparation of Acetone Powder

Minced muscle is extracted with sol. B for ten minutes at 4°C, diluted with four volumes of cold distilled water and filtered. The liquid is squeezed out of the muscle through muslin. The tissue is resuspended in four volumes (w/v) of solution C and stirred for 20 min. at 4°C. It is again filtered and squeezed. The residue is minced, and suspended in one volume of solution D, stirred for ten minutes (4°C) and diluted 10:1 with solution E. After stirring for ten minutes, it is filtered and squeezed. The tissue is resuspended in three volumes of cold acetone, filtered and squeezed and the step repeated with one volume of acetone, leaving the suspension for 20 minutes before filtering. Finally the muscle is suspended in one volume of acetone with 5 ml of Na_2CO_3 1^{-1}, filtered, squeezed and spread out to dry overnight (or 15 hours) on filter paper.

Purification of Actin

The acetone powder is extracted at 0°C for 30 min. in solution A (200 ml per 10 g powder) and filtered through a coarse sintered funnel. The residue is washed in 100 ml of solution A and the combined supernatents centrifuged at 10,000 g for 1 hour. Solid KCl is added to the supernatent to a final concentration of 50 mM, and $MgCl_2$ to 2 mM. The actin is left to polymerise for two hours. KCl is added to 0.6 M, with gentle stirring, and left to stir for 90 min. before centrifugation at 80,000 g for 3 hours. The pellet is suspended in 30 ml of solution A, and dialysed for three days against solution A. After cleaning by centrifugation (80,000 g, 3 hrs), the actin is polymerised as above. The final cycle from the first to the second polymerisation is repeated. It can be stored at 4°C with a crystal of thymol, and dissolved in 0.6 M KCl before use in actomyosin threads. Average yield is 200 mg from 10 g of powder.

Preparation of Troponins-Tropomyosin

The residue from the actin, acetone powder is extracted in 10 mM Tris pH 8.3, 0.5 mM 2 mercaptoethanol for 3 hours, and centrifuged at 16,000 g for 1 hour. 35.4 g of ammonium sulphate per 180 ml are added and stirred for 20 min. ($4^{o}C$). After centrifugation at 16,000 g for 30 min., 54 g of ammonium sulphate are added per 180 ml and the previous centrifugation step repeated. The pellet is dissolved in 20 ml of 10 mM Tris pH 8.3, 0.9 mM 2 mercaptoethanol, and dialysed against the same overnight with three changes of buffer. It is then cleaned by centrifugation at 90,000 g for 2 hours. Proteins were exhaustively dialyzed against 0.6 M KCl before use in actomyosin threads.

Natural actomyosins were used on the day of preparation and purified proteins directly following preparation. Actin:Myosin:tropomyosin: (troponins) were mixed at high ionic strength in the molor ratios 7:1:1: (1:1:1) (after Potter, 1974). Protein concentrations varied between 50-65 mg ml^{-1} as determined by a microbioret method (Itzhaki and Gill, 1964).

ATPase Activities

Actomyosin ATPase activities were determined in a medium containing 50 mM imidazole, 40 mM KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 2.5 mM ATP and protein concentrations of 0.1 - 0.5 mg ml^{-1}. Incubations were initiated by the addition of the ATP, and stopped with 1 ml of 10% TCA per ml of incubation medium. Assays were carried out at $37^{o}C$ (pH 7.0) for rabbit, and $15^{o}C$ (pH 7.4) for dogfish actomyosins. Phosphate release is measured spectrophotometrically by the formation of a coloured complex with ammonium molybdate and iron sulphate (Rockstein and Herron, 1951).

Electron Microscopy

Activated and relaxed threads were prepared for electron microscopy by fixation in 6% glutaraldehyde (in 0.15 M phosphate buffer pH 7.2),

dehydration through a series of alcohols, and embedding in araldite (CY212 Resin, EM Scope, England). Thin sections (20-30 μm) were cut (Reichert UMU2 ultramicrotome), stained with 2% uranyl acetate and counterstained with 0.4% lead citrate. Formvar coated copper grids (see below) were used to give support to sections. Sections were examined under a Philips EM301 electron microscope. Threads were also disrupted by gentle homogenisation, and negatively stained with 2% uranyl acetate prior to examination. Formvar coated grids are made in the following way. Freshly prepared 3-6% Formvar in clean, dry chloroform is used. Clear glass slides are dipped momentarily into the solution and left to drain for 5 minutes in a covered jar, containing an atmosphere saturated with chloroform. The slides are then allowed to dry in a clean dust free atmosphere. A rectangle is scored into the coating, and floated off onto distilled water. Upturned copper grids are placed on the formvar film. The film is then 'flipped' off with a glass slide, and the grids left to dry out before use.

SDS-gel Polyacrylamide Electrophoresis

The method is modified from that of Potter (1974). Freeze dried samples of protein are dissolved at 1-2 mg ml^{-1} in 8 M urea, 0.4% SDS, 0.17 M Tris pH 6.3 and 1% 2 mercaptoethanol. Gels have the following composition: 12.5% acrylamide, 0.3% bis-acrylamide, 8 M urea, 0.1% SDS, 0.375 M Tris pH 8.6, polymerised with 61 μl TEMED (NNN'N'-tetramethyl ethylene diamine) and 11 mg ammonium persulphate. 10 cm tube gels are run, with a buffer of 25 mM Tris pH 8.6, 80 mM glycine, 0.1% SDS. Gels are given a prerun of 2 mA per tube before being loaded with 5-20 μl of the protein samples and standard proteins of known molecular weight (12,000-70,000 daltons (Sigma)) bromophenol blue markers. They are then run at 1 mA per tube for the first hour, and then at 2 mA per tube until one hour after the marker dye runs off the end of the gels. A one hour

stain in 0.15% Coomassie Brilliant blue in 5% acetic acid, 40% methanol is used before destaining in 5% methanol, 5% acetic acid, and storage in 7.5% acetic acid. Gels were scanned on a Joyce-Loebl Chromoscan Mk II scanning densitometer.

Experimental Protocol

The solubilised protein is loaded into 1 ml plastic syringes with specially prepared needles (inside diameter = 240 μm). The tips are removed, and the walls bevelled to decrease turbulence during extrusion. The needles are bent through 90° at their mid-point. A long shallow trough is filled with a solution containing 50 mM KCl; 20 mM imidazole (pH 7.0); 5 mM $MgCl_2$ and 0.1 mM DTT (or DTE, or mercaptoethanol). The plunger is depressed slowly as the syringe is drawn quickly through the trough, the needle pointing away from, and parallel to, the direction of movement. With practice uniform threads of precipitated protein can be extruded, many centimetres long. Precipitation is not instantaneous, the threads increase in opaqueness over the first 20 seconds or more. For this reason they are left in the above extrusion solution for 5 minutes prior to use. 5 mm long sections are excised and attached to the transducer hooks, the top hook being held by adjustable stops. The two ends are glued to the bottom hook with a plexiglass acetone glue, the loop passing over the top hook. Threads are quickly immersed in a solution containing 50 mM imidazole (pH 7-7.4), 40 mM KCl, 5 mM EGTA. 5 mM $CaCl_2$, 3 mM $MgCl_2$, 0.5 mM $Na_4P_2O_7$. An ATP regenerating system is also included - 10 mM creatine phosphate and creatine kinase at > 20 units ml^{-1}. The incubation medium is contained in small, perspex baths, which can be raised on a micromanipulator to immerse the thread. A microstirrer is used to mix the solution continuously. Temperature can be controlled to within 0.1°C by passing a coolant through the perspex chamber surrounding the baths. The adjustable stops are removed from

the top hook and the tensiometer set in the isometric mode. It has been calculated (Crooks and Cooke, 1977) that diffusion of creatine phosphate and creatine kinase to the centre of a 400 μM diameter thread will take approximately 2 and 4 minutes respectively. The threads are left for 8 minutes prior to activation. ATP, added to final concentration of 500 μM, is used to initiate contraction. In some experiments, $CaCl_2$ is omitted from the incubation medium, and used as the activator. Activation is normally complete within 2-5 minutes. As soon as tension is stable, a force velocity curve can be obtained. Maximum, unloaded contraction velocity was first measured by setting the calibrated 10 turn potentiometer to zero. The apparatus is switched to isotonic, and the thread released, then returned to L_0 in isometric. While in isotonic, the voltmeter reading is the isotonic load. This is repeated at increasing loads until contraction velocity falls to zero. Unloaded contraction velocity is then measured again. If it has fallen by more than 5% of the original value, the data are rejected. Threads can maintain tensions for many minutes, occasionally up to one hour.

RESULTS

Purity of Natural Actomyosins

Densitometric scans of rabbit natural actomyosins are shown in Figure 3:4. Actin, myosin, myosin light chains and the Ca^{2+} regulatory proteins are present in stoichiometric ratios similar to those reported in the literature (Potter, 1974; Lowey and Risby, 1971).

Electron micrographs of araldite embedded sections and disrupted threads are shown in Figure 3:5. F actin decorated with myosin "arrowheads" can be seen. No contamination with membrane fragments was observed. At the high protein concentrations used in the mechanical experiments, the packing of filaments was too dense to allow a detailed

examination. However, a high degree of parallel alignment of filaments along the long axis of the thread was noted in activated threads (D'Haese and Komnick, 1972a,b).

Mechanical Results

The initial aim was to assess the usefulness of actomyosin threads for comparative studies on muscle. For this reason the isometric and force-velocity parameters of actomyosin were determined on threads prepared from muscles with known differences in their physiological contraction velocities.

Figure 3:6 shows typical activation curves from natural actomyosin threads prepared from dogfish (Scyliorhinus canicula) red (slow) and white (fast) muscle, and from rabbit ventricle and back (fast skeletal) muscle. Times to half maximal tensions are 96 s and 66 s for dogfish white and red, and 63 s and 48 s for rabbit skeletal and cardiac threads, respectively.

Figure 3:7 shows force-velocity data taken from 14 white threads (from six animals), and six red threads (three animals) from dogfish muscle. Velocities are in thread lengths s^{-1}, P/P_0 = applied force/maximal isometric tension. Temperature = 15°C, pH 7.4. Error bars indicate ± 1 s.d.

Similar data are shown in Figure 3:8 from six skeletal and seven cardiac threads from the rabbit (each from three animals); experiments done at 37°C pH 7.0.

Figure 3:9 shows a typical curve for dogfish red actomyosin, plotted according to the linear form of Hill's equation (Hill, 1939). All threads analysed in this way gave a straight line up to values of 0.7 P_0.

A small number of experiments were performed on reconstituted actin, myosin and tropomyosin-troponins, purified from rabbit skeletal

muscle. A typical force velocity curve is shown in Figure 3:10 for comparison with the natural actomyosin curves. Conditions as for rabbit natural actomyosin experiments.

Mean unloaded contraction velocities, maximal isometric tensions and actomyosin ATPase activities are summarised in Table 3:1 (mean ± 1 S.D.).

DISCUSSION

The data reveal several unphysiological characteristics. First (in agreement with previous studies), maximal isometric tensions measured (Figure 3:6) are very much smaller (around 10 g cm^{-2}) than those obtained from intact fibres (2-3 kg cm^{-2} (Gordon et al., 1966)) and skinned fibres (1.4-1.7 kg cm^{-2} (Hellam and Podolsky, 1969)). Second, velocities are all less than 0.014 Ls^{-1} (Figures 3:7, 3:8, 3:1), two to three orders of magnitude lower than those of intact muscle fibres. For example, V_{max} values for Tilapia adductor operculi white and red fibres are 2.6 and 1.5 Ls^{-1} at 18°C (Flitney and Johnston, 1979). Lannergren (1978) reports values of 6.3 and 1.1 Ls^{-1} for Xenopus fast and slow fibres (22.5°C) and Close (1969) gives values of 14.5 and 6.3 Ls^{-1} for whole extensor digitorum longus and soleus muscles of the rat (35°C). Third, the relative velocities of the different thread types do not reflect those of the corresponding muscles (Figures 3:7, 3:8). Barany (1967) has shown a positive correlation between the actomyosin ATPase activity and the contraction velocity of a muscle. However, the velocities of the threads from both animals show a complete reversal of the results predicted from both the ATPase activities and the studies noted above on single fibres and whole muscles from different muscle types.

To take up the first point, a number of factors are likely to

contribute to the small tensions. Protein concentrations in the threads used in the above experiments varied from 50-65 mg ml^{-1}. Crooks and Cooke (1977) used concentrations of 15-45 mg ml^{-1}. They showed that tension is proportional to protein concentration. If their results are extrapolated to a protein concentration similar to that estimated for muscle of 1.2×10^7 M^2 (based on Tregear and Squire (1973)), then the tensions produced by natural actomyosins would be around 340 g cm^{-2}. Ultrastructural studies by D'Haese and Komnick (1972a,b) on extruded actomyosin threads have shown that they lack the very ordered filament arrangement found in intact muscle. Relaxed threads contained a random network of actin filaments, decorated with myosin molecules and oligomeric myosin bridges. Stretching relaxed threads led to a degree of orientation of the actin filaments along the long axis. Isotonic contraction brought about by the addition of ATP led to a condensation of the actin network and the formation of myosin filaments. Isometric contraction again led to the formation of myosin filaments, but there is a marked alignment of both filament types with the long axis of the thread. The organisation of filaments within muscle ensures that the actin-myosin interaction occurs with the correct polarity. If the interfilament interaction in threads is random, only half will have the correct polarity for force generation. This would suggest that the extrapolated estimate given above should be half the expected value for intact muscle - i.e. an estimated value for muscle would be 700 g cm^{-2}. This is still only half the measured value for skinned fibres, but the data does suggest the tensions generated by actomyosin threads are produced by an actin-myosin interaction similar to that of intact muscle. The absence of the correct actin-myosin interaction polarity could also explain the very low velocities. Contraction velocity in muscle is determined by the intrinsic cross-bridge cycling rate, the sarcomere length, and the length of the muscle itself.

By expressing velocity in muscle lengths per second, the latter need not be considered. It is unlikely that the cross bridge cycling rates of the myosin filaments would be drastically altered in actomyosin threads, the AM.ATPase activities of threads are similar to myofibrillar ATPase activities. The most important factor determining velocity is probably therefore related to the geometry and packing of filaments within the threads. Cooke and Franks (1978) found velocity to be proportional to isometric tension. As stated above, tension is proportional to protein concentration. The closer packing of the filaments at high protein concentrations therefore increases contraction velocity. The highly organised hexagonal arrangement of actin and myosin filaments in T.S. and into sarcomeres in L.S. do not permit such an effect in muscle since protein content in a myofibril is fixed. This is not the case in actomyosin threads. D'Haese and Komnick (1972a,b) produced threads by two methods. First, by rapid extrusion and precipitation of a solution of actomyosin. Relaxed threads contained actin filaments, and myosin molecules/oligomeric myosin bridges (see above). Second, by extrusion of actomyosin which had been dialysed, to precipitate the myosin slowly. Relaxed threads contained both actin and myosin filaments. The initial unloaded contraction velocities of these threads was over four times greater than those of rapidly precipitated threads. Contraction velocity thus seems to be very dependent upon filament dimensions.

Other studies have shown that filament formation by purified rabbit myosin depends to a large extent on the conditions of formation, and the age of the myosin. The length, diameter and shape of synthetic filaments are affected by pH (Josephs and Harrington, 1966; Kaminer and Bell, 1966) ionic strength (Kaminer and Bell, 1966), and the concentrations of ATP, PO_4^{3-}, Mg^{2+} and Ca^{2+} (Pinset-Härström and Truffy, 1979). Specific concentrations of ATP and Mg^{2+} are needed for the formation of

filaments with physiological characteristics (Pinset-Härström and Truffy, 1979). Filament length was greatest at pH 6.5, with dilution from 0.6 to 0.1 M KCl over 3 minutes. Increasing final ionic strength and/or pH decreased filament length, tapered ends were lost, and filaments became irregular in shape (Kaminer and Bell, 1966). In the presence of equal millimolar amounts of ATP and Mg^{2+}, Pinset-Härström and Truffy produced filaments 5-15 μm in length, apparently built up of 2-3 nm wide subunits parallel to the long axis. Overall diameter was 15-17 nm. The filaments possessed tapered ends, and 60 nm filamentous projections pointing systematically in the direction of the tapered ends. Central zones of polarity reversal were easily identified. Storage of myosin at 4°C results in a loss of the ability to form these "physiological" filaments. Over a two week period, there was a continuous decrease in length and increase in diameter, with the loss of the bipolarity. Within four days, mean length fell from 10 μm to 5 μm, and to < 2 μm in seven days. Accompanying this trend is a loss of the LC_2 light chain. Removal of this light chain from fresh myosin by treatment with dithio-bis-nitrobenzoic acid (DTNB) mimics this process.

Structural differences amongst the myosins of fast, slow and cardiac muscles are well documented (Syrovy, 1979). For example, fast muscle myosins are more susceptible to trypsin digestion than slow muscle myosins (Gergely et al., 1965). Myosins show differences in their ATPase activation patterns by SH reagents (Sreter et al., 1966), and Weeds and Burridge (1975) distinguished fast and slow HMM's on differences in their cysteine containing peptides after trypin digestion. Fast HMM has a characteristic 88,000 dalton peptide after digestion, absent from slow muscle digests (Jean et al., 1975). Fast rabbit myosin also contains 1.8 mol mol^{-1} of β-methylhistidine, none has been detected in slow myosin (Kuehl and Adelstein, 1970; Johnston and Perry, 1970). Photo-oxidation of this molecule does not change ATPase activity,

but it may have a structural role (Johnston and Perry, 1970). Differences have also been observed with non-chemical techniques. Nakamura et al. (1971) demonstrated differences in staining in LMM paracrystals, reflecting structural dissimilarities in the myosin backbone. Weeds and Lowey (1971) characterised three light chains in skeletal muscle, a 'DTNB' light chain LC_2, and two 'alkali' light chains, LC_1 and LC_3, and SDS gel electrophoresis has shown characteristic differences between these and light chains from slow muscles (Lowey and Risby, 1971). Weeds and Pope (1971) found differences in the structure of the two light chains present in cardiac myosin as compared to skeletal. It has already been noted that the LC_2 light chain can affect filament formation. It is possible that different myosins will differ in their abilities to form filaments under the conditions required to prepare and activate threads and this is responsible for the discrepancies seen between thread contraction velocities on the one hand and contraction velocities of intact fibres, and AM-ATPase activities on the other.

Calcium sensitivity (as defined by Lehman and St. Gyorgyi (1975)) in the threads was difficult to achieve, even though ATPase activities of the actomyosins were typically > 80% calcium sensitive. SDS gel electrophoresis confirmed the presence of the calcium regulatory proteins in the threads of natural actomyosins. In the absence of Ca^{2+}, threads usually began to contract slowly upon the addition of ATP. The addition of Ca^{2+} led to an increase in the rate of tension development. A similar effect was reported by Wideman, Maruyama and Hayashi (1970) on rabbit, skeletal, natural actomyosin. Attempts to relax Ca^{2+} sensitive threads have only been partially successful. Crooks and Cooke (1977) and Wideman et al. (1970) added sufficient EGTA to effectively bind all free Ca^{2+}, and tensions fell on average by only 50% over 10 minutes. Wideman et al. report relaxation ranging from 15-100% over 12 experiments.

In conclusion, our results, in conjunction with the above studies, suggest that filament formation, geometry and packing, and not differences in XB cycling rates, largely determine the observed properties of actomyosin threads. Thus, any physical or chemical manipulation (such as protein concentration, storage time, ion concentrations, DTNB treatment) of the actomyosin that alters its ability to form filaments is likely to result in secondary, unphysiological changes in the contractile properties of threads.

Figure 3:1: Tensiometer. Details of the lever and photodiode assembly. C = coil on light, foil former; F = foil flag; H = platinum hook; I = infra-red LED; L = tubular aluminium lever; M = magnet; MM = micromanipulator; P = diamond pivot; Ph = photodiode; S = adjustable stops.

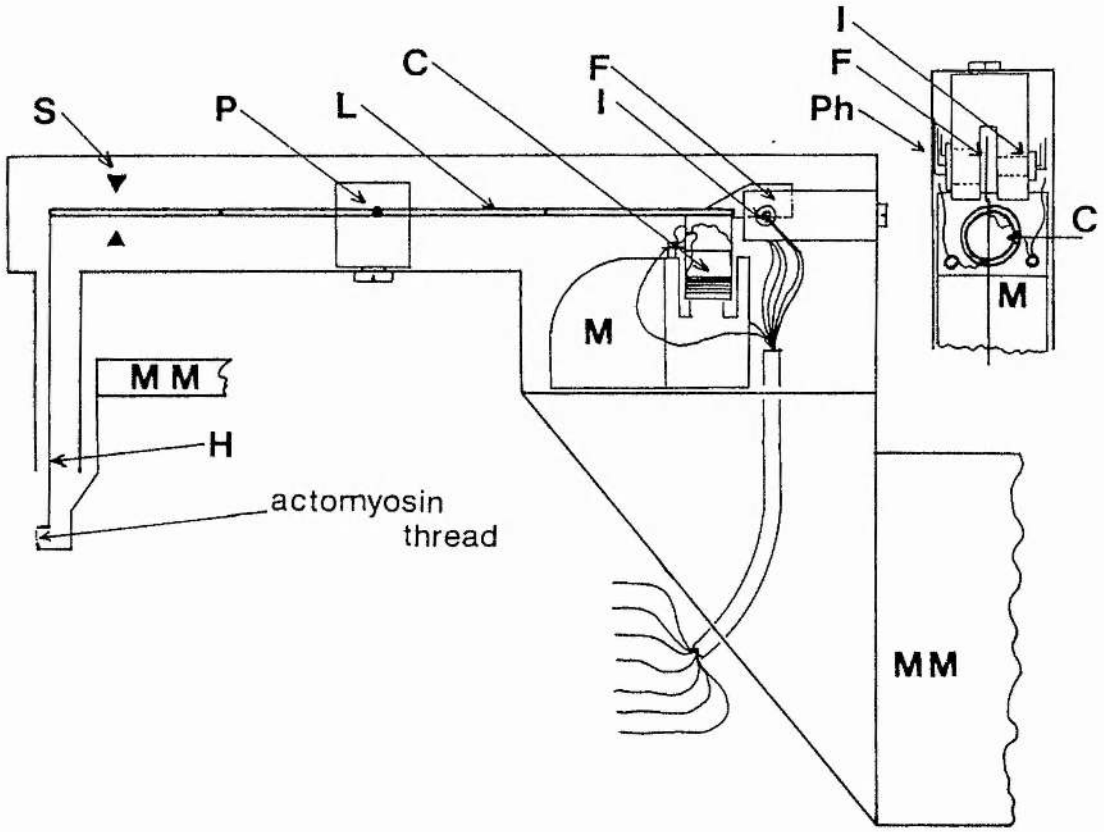

S
P
L
C
F
I
Ph
F
I
C
M
M
M M
H
actomyosin
thread
MM

Figure 3:2: Tensiometer. Block diagram of apparatus.

C = coil on light foil former; I = infra-red LED;

L = tubular aluminium lever; Ph = photodiode.

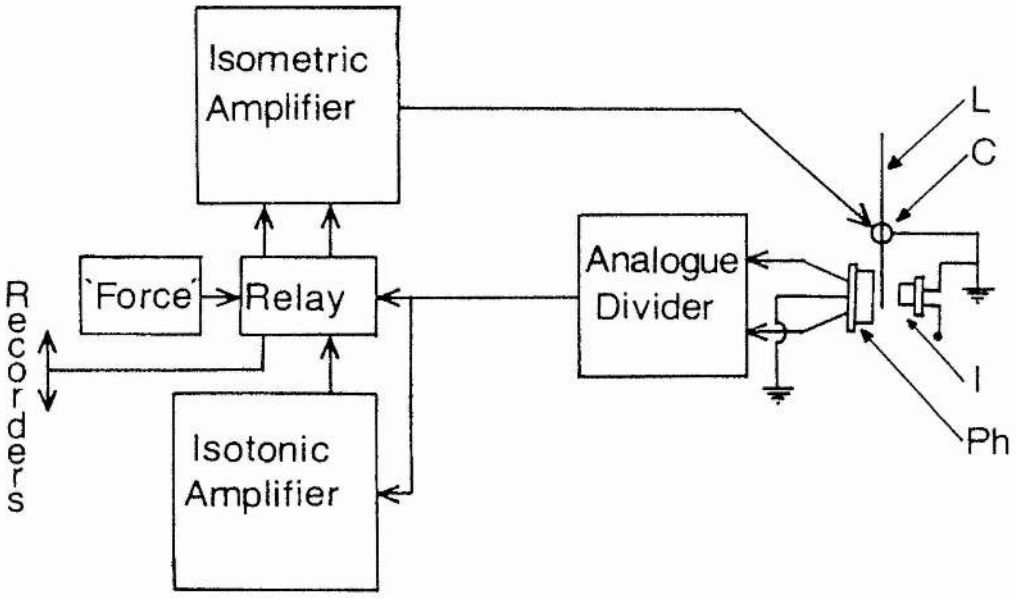

Isometric
Amplifier
L
C
Analogue
Divider
`Force´
Relay
Recorders
I
Ph
Isotonic
Amplifier

Figure 3:3: Tensiometer. Circuit diagram.

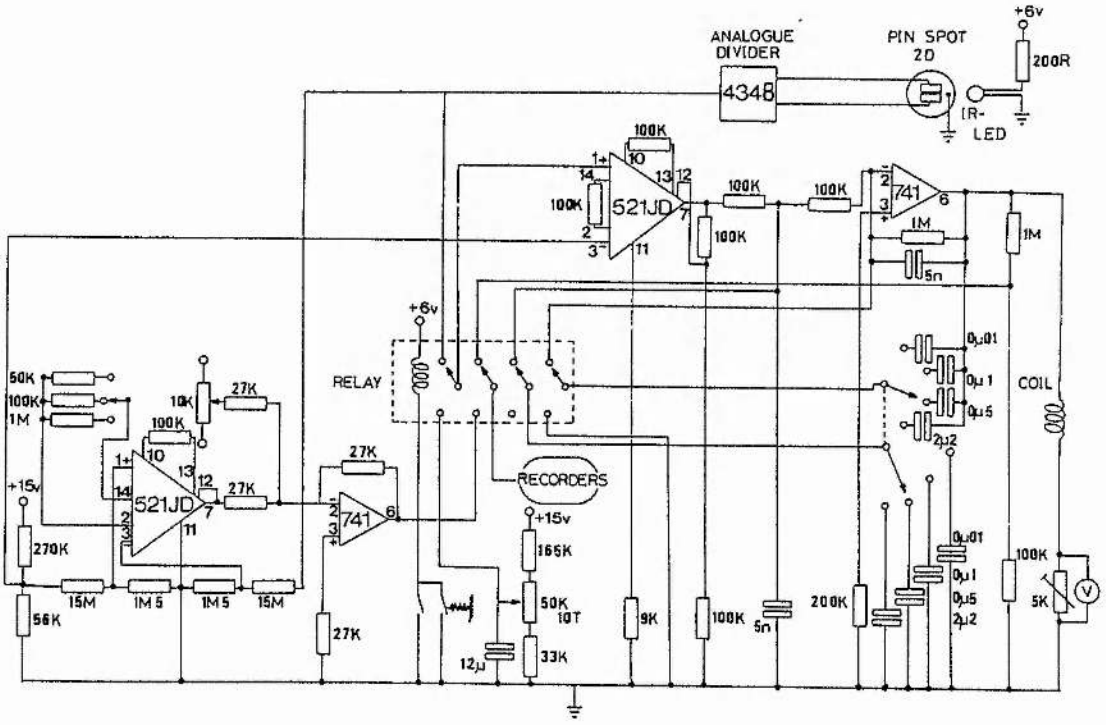
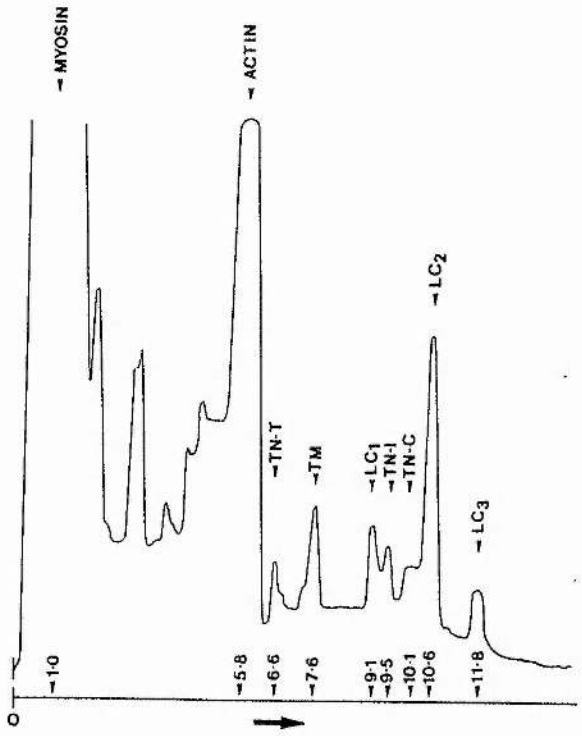

ANALOGUE DIVIDER
4348
PIN SPOT 20
+6v
200R
R-LED
100K
521JD
741
1M
5n
RELAY
RECORDERS
+15v
50K
100K
1M
27K
10K
270K
56K
15M
1M5
165K
50K
10T
33K
12µ
9K
200K
0µ01
0µ1
0µ5
2µ2
COIL
5K

Figure 3:4: Densitometric scans of urea-SDS polyacrylamide gels.

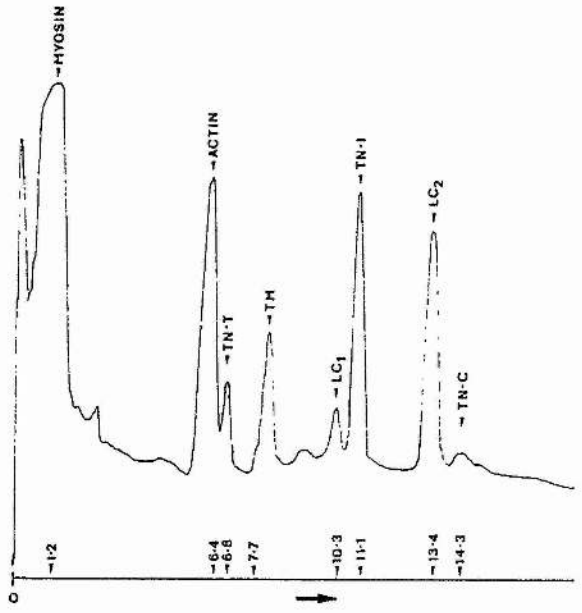
(a) Rabbit skeletal natural actomyosin;

(b) Rabbit cardiac natural actomyosin.

Molecular weights (daltons). Skeletal: Myosin = 500,000; Actin = 44,700; Troponin-T (TN-T) = 38,000; Tropomyosin (TM) = 35,000 (X2); Myosin light chain 1 (LC_1) = 25,000; Troponin-I (TN-I) = 24,000; Troponin-C (TN-C) = 17,800; LC_2 = 18,000; LC_3 = 16,000. Cardiac: LC_1 = 27,000; LC_2 = 20,000. Large arrows = direction of migration, numbers refer to distance travelled from origin (cm).


MYOSIN
ACTIN
TN-T
TM
LC1
TN-I
TN-C
LC2
LC3
1·0
5·8
6·6
7·6
9·1
9·5
10·1
10·6
11·8
0

a

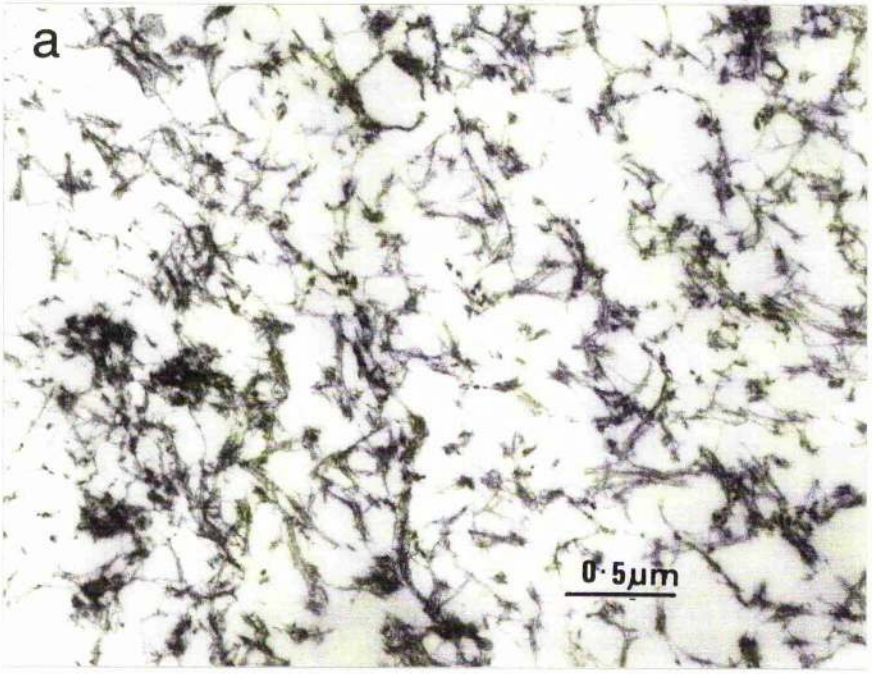

HYOSIN
ACTIN
TN-T
TM
LC1
TN-I
LC2
TN-C
1·2
6·4
6·8
7·7
10·3
11·1
13·4
14·3
0

b

Figure 3:5: Electron micrographs of actomyosin threads.

(a) Araldite embedded section of low protein concentration (15 mg ml^{-1}) thread;

(b) Negatively stained "decorated" actin filaments from homogenised thread.


a
0·5µm

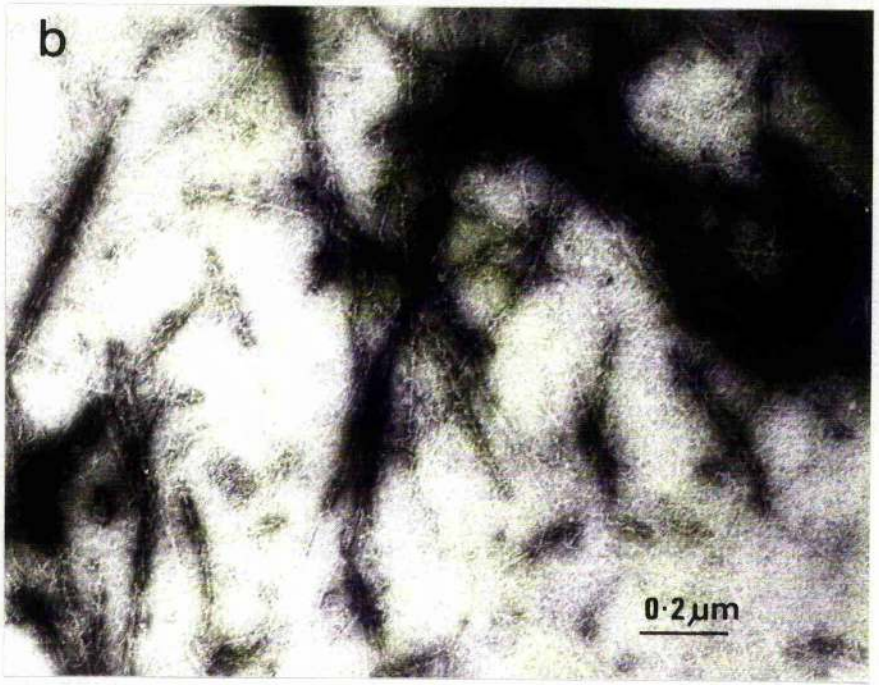

b
0·2µm

Figure 3:6: Development of isometric tension with time in actomyosin threads activated with ATP.

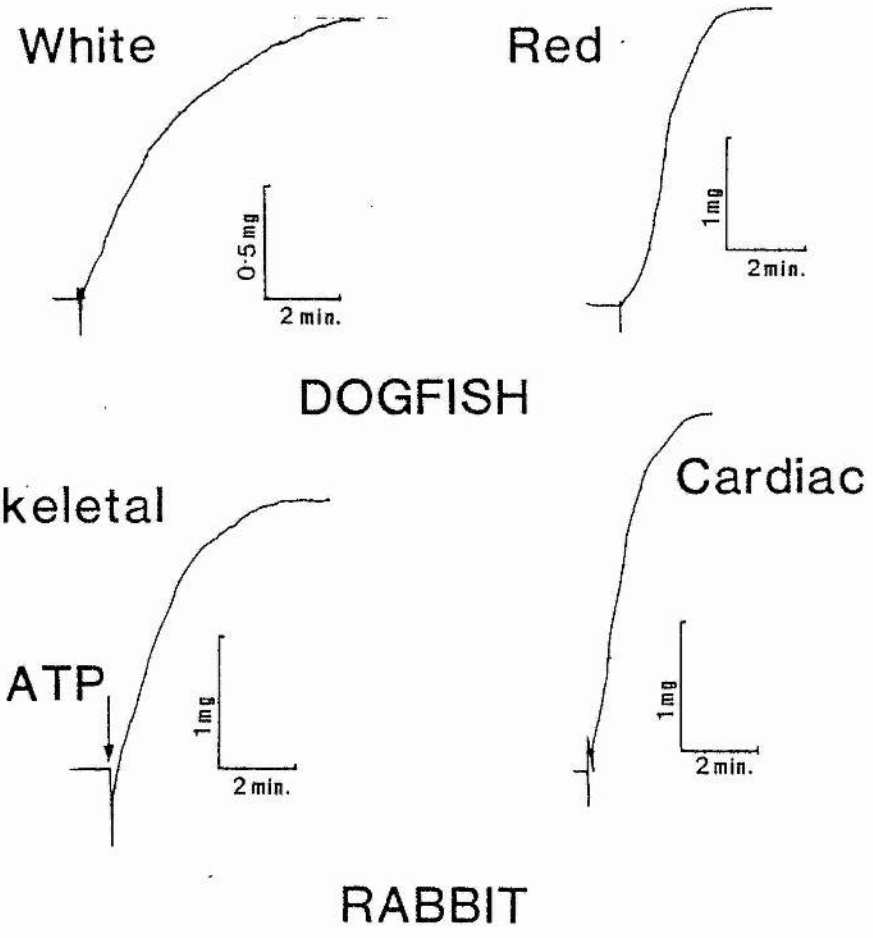

White
0·5mg
2 min.
Red
1mg
2min.
DOGFISH
Skeletal
ATP
1mg
2 min.
Cardiac
1mg
2min.
RABBIT

Figure 3:7: Force-velocity relationships for dogfish threads at 15°C. ○, red muscle; ●, white muscle. Velocities are in thread lengths per second. P/P_0 = force applied/maximal isometric tension. Error bars indicate ± 1 s.d.

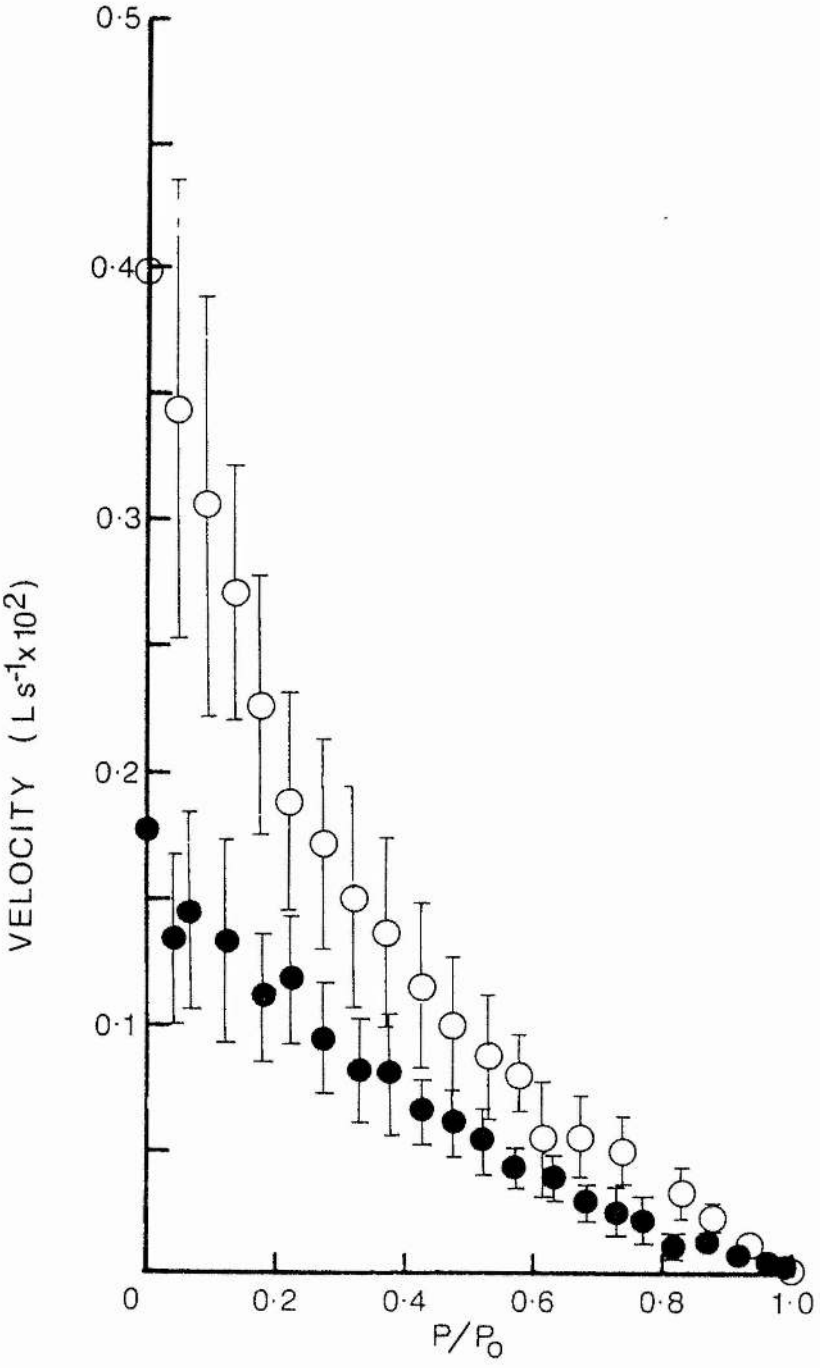

0·5
0·4
0·3
0·2
0·1
0
VELOCITY (L s⁻¹ x 10²)
0·2
0·4
0·6
0·8
1·0
P/P₀

Figure 3:8: Force-velocity relationships for rabbit threads at 37°C. Open symbols represent seven individual cardiac threads, from three animals, solid symbols six threads of fast skeletal muscle, also from three animals.

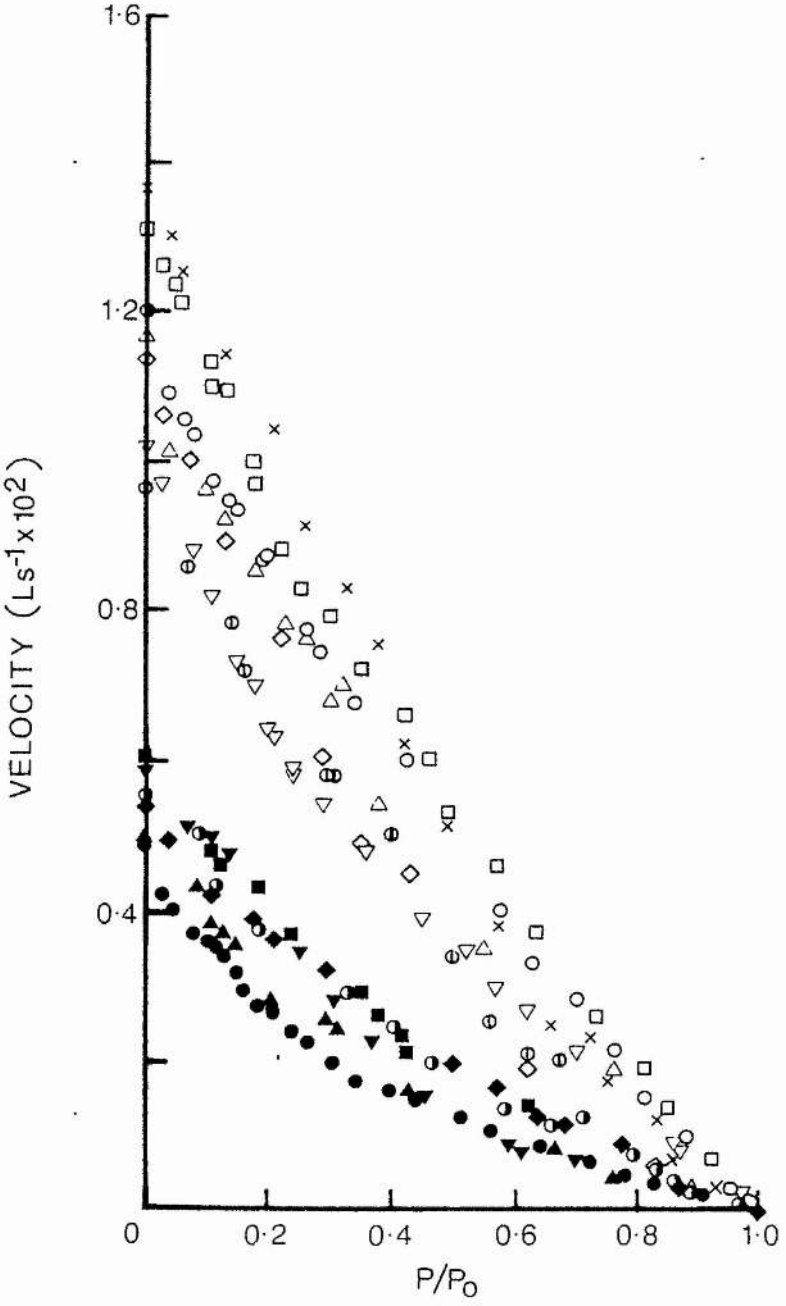

1·6
1·2
0·8
0·4
0
0·2
0·4
0·6
0·8
1·0
VELOCITY (Ls^{-1}x10^2)
P/P$_O$

Figure 3:9: Typical red dogfish curve plotted according to the linear form of Hill's equation, $(P+a)v = b(P_0-P)$, where a and b are constants of proportionality. Points below 0.7 P_0 lie on a straight line. A similar fit has been described for intact single fibres (Lannergren, 1978).

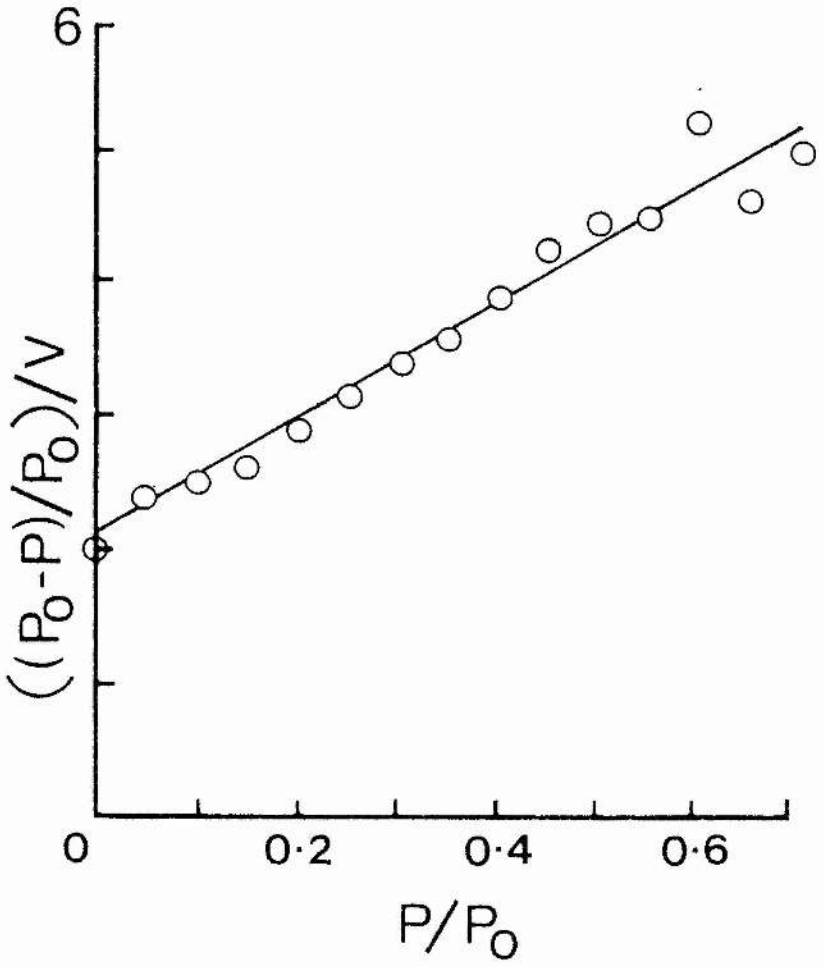

6
$((P_O-P)/P_O)/V$
0
0·2
0·4
0·6
P/P_O

Figure 3:10: Force velocity curve for a thread prepared from purified actin, myosin and regulatory proteins of skeletal muscle.

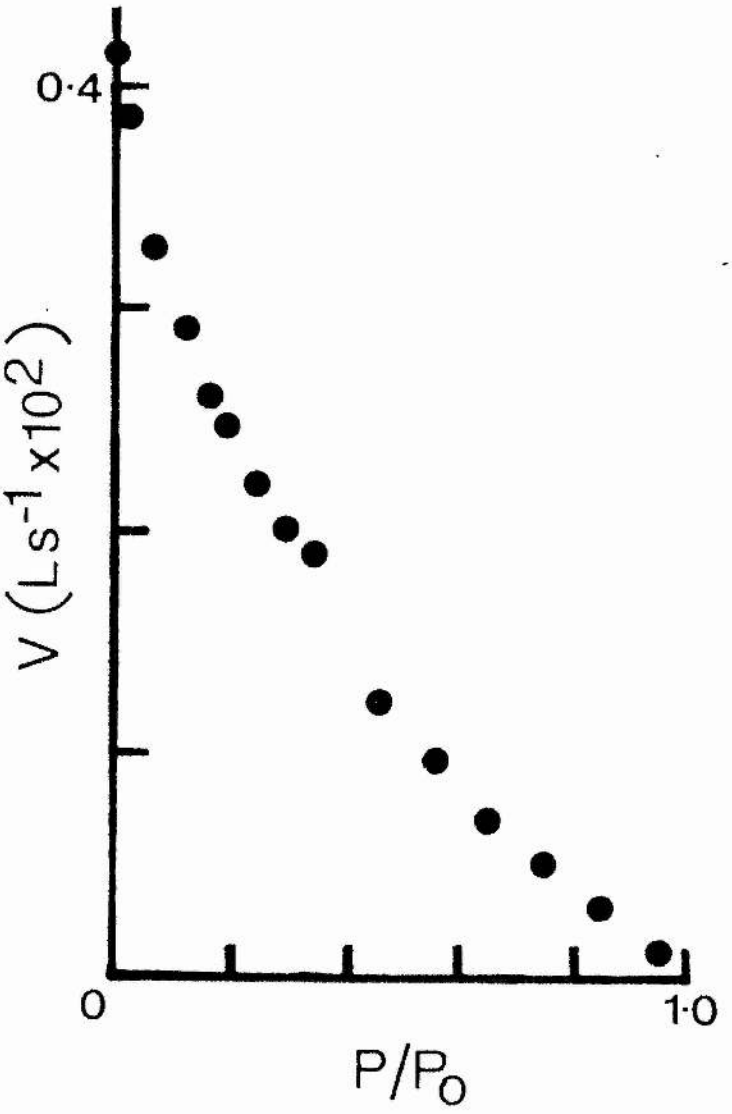

0·4
V (Ls^{-1} x10^2)
0
1·0
P/P$_O$

Table 3:1: Summary:

Ls^{-1} = thread lengths s^{-1}; errors indicate ± 1 S.D. ATPase activities were determined in an incubation medium of 50 mM imidazole, 40 mM KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 2.5 mM ATP, protein concentration 0.1 to 0.5 mg ml^{-1}; rabbit activities were measured at 37°C, pH 7.0, dogfish activities at 15°C, pH 7.4.

Actomyosin source	Mean V_{max} ($L\ s^{-1} \times 10^2$)	Mean P_0 ($g\ cm^{-2}$)	Actomyosin ATPase activity ($\mu M\ P_i\ mg^{-1}\ min^{-1}$)
Rabbit			
Skeletal	0.53 ± 0.05	59.8 ± 17.2	0.51
Cardiac	1.16 ± 0.14	67.1 ± 16.8	0.07
Dogfish			
White	0.18 ± 0.06	26.0 ± 9.7	0.55
Red	0.39 ± 0.12	57.0 ± 4.1	0.18

CHAPTER 4

APPARATUS FOR STUDYING THE ISOMETRIC AND FORCE-VELOCITY CHARACTERISTICS OF SKINNED FIBRES

INTRODUCTION

The earliest experiments to investigate the relationship between contraction velocity and load were performed by A. V. Hill in 1922. Hill measured the maximum speed of shortening of human arm muscles in subjects pulling against inertial loads. Similar experiments were performed two years later by Gasser and Hill (1924) on isolated frog sartorius muscles. However, most of these early experiments were concerned more with the investigation of the total work evolved as a function of load. In the 1930s, the emphasis moved to the relationship between velocity and load, with the experiments of Stevens and Metcalf (1934) and Fenn and Marsh (1935). In 1938 Hill conducted his elegant experiments which led to a mathematical formulation describing the force-velocity relationship. He demonstrated that the muscle developed a greater amount of heat whilst shortening than when held at constant length. This extra heat was proportional to the total shortening, with a constant of proportionality he called a. The mechanical power = Pv, where P = load, v = velocity, and the rate of heat production = aV. Thus, the rate of release of energy during contraction = (P+a)v. This was shown to be linearly related to the load on the muscle:

$$(P+a)v = b(P_0-P) \ ,$$

where P_0 = maximum isometric tension and b is a constant. Hill demonstrated a close agreement between the results he obtained from thermal and mechanical measurements.

The mechanical apparatus used on isolated frog sartorius muscles by Hill consisted of an aluminium lever mounted on ball bearings, the free end of the muscle being attached to one side of the lever. Step tension releases could be applied during an isometric contraction by releasing the lever electromagnetically, allowing the muscle to contract against loads suspended from the lever, on the other side of the fulcrum. By careful design compliance and inertia in the system were minimised.

The simple principle of isotonic release has since been used by many workers to study FV characteristics, first on whole muscles (e.g. Jewell and Wilkie, 1958; Close, 1964; Woledge, 1968), and later on small fibre bundles (Civan and Podolsky, 1966) and single fibres (Edman et al., 1976; Edman, 1979; Lannergren, 1978, 1979). The technique has also been applied to skinned fibre preparations (Podolsky and Teichholtz, 1970; Wise et al., 1971; Gulati and Podolsky, 1978). The low loads required for work on single and skinned fibre preparations have been achieved by the use of small spiral and helical springs under tension (e.g. Edman et al., 1976; Lannergren, 1978). One advantage of this system is the low inertial forces on the lever - an important consideration when the tensions involved are generally less than 200 mg.

Advances in technology have led to the development of more versatile apparatus: the use of rapid servo-systems allows accurate "clamping" of either length or tension by negative feedback. A. F. Huxley and Simmons have been able to effect step tension changes in < 1 ms. (A. F. Huxley, 1971). For technical reasons, however, high-time-resolution kinetic studies are now usually performed with step length changes (e.g. Huxley and Simmons, 1971). The rapid step load change, followed by force clamping, has been successfully employed to measure contraction velocity in skinned fibres (Julian, 1971; Julian and Moss, 1981).

The system described below is a variation on the isotonic lever

design. Rather than applying force mechanically, however, the lever is built around a sensitive moving coil galvanometer, and force is adjusted by varying the current in the coil. The system therefore has very little inertia, there being no appreciable mass away from the fulcrum. A second advantage is that fibres can be returned electronically to L_0 in a slow, controlled fashion. In common with many of the systems noted above, displacement is measured photoelectrically, and force with an isometric strain guage.

MATERIALS AND METHODS

Apparatus

The apparatus developed for these studies allows the independent measurement of tension and length. A block diagram is shown in Figure 4:1, and details of the mechanical section in Figure 4:2. A section of skinned fibre 1-2.5 mm in length is glued between the two glass hooks. One hook is attached directly to the silicon beam of an AE803 strain guage element (A.M.E., Horton, Norway). The element is held rigidly in a screened, aluminium and perspex adaptor, mounted on a one way micromanipulator. This allows the distance between the hooks to be adjusted. The output from the element is fed to a bridge circuit and amplifier unit. The sensitivity varies slightly with the element in use, a typical value would be 3.5 mV mg^{-1}. Noise is < 3 mV, drift < 1 mV hr^{-1}. The other hook is attached to a 3.7 cm long balsa wood lever. During isometric contractions, a brass pin, glued to the armature of a miniature relay, holds the free end of the lever against a stop. The total compliance of the system is < 40 $\mu m\ g^{-1}$.

The other end of the lever is attached to a meter movement taken from a sensitive moving coil galvanometer. The isotonic afterload is generated by passing a current through the coil, to produce forces of

0 to > 250 mg. This is displayed as a voltage on a digital voltmeter. Voltage is linearly proportional to load. Activation of the relay draws back the brass pin, allowing a contracting fibre to shorten. The equivalent mass of the lever is < 20 mg. A delay circuit is built into the system between the trigger and the relay. The oscilloscope, triggered directly, then shows the tension and length traces for 50 ms prior to the release.

During an isotonic release, an aluminium flag attached to the lever crosses the path of an L.E.D.-photodiode assembly similar to that described in Chapter 3. Using similar amplification circuits, displacement against time can be measured. Sensitivity = 43 μm/100 mV. After a release, by feeding the photodiode current back into the coil, with suitable electronic damping, the lever can be brought slowly back to its stop, and the relay deactivated. A second stop is placed in front of the fibre - the total shortening allowed is 400 μm.

A block of perspex mounted on a two way micromanipulator slides in a channel beneath the hooks. The lower half contains circulating ethylene glycol/water from a Grant cooling system, to regulate temperature (± 0.5^{o}C). The upper half has three chambers, each of 1.5 ml capacity. The block can be raised to immerse the fibre in any of these three chambers. A change from one bath to another can be effected in < 5 s. Thin perspex windows set in the block allow the beam from a He-Ne laser to be passed through the fibre from below. The diffraction pattern is viewed on a small translucent screen placed above the fibre. The screen is calibrated in sarcomere lengths, calculated from Bragg's equation for diffraction:

$$s = \frac{\lambda}{\sin \theta}$$

where s = sarcomere length, λ = wavelength of laser (0.6328 μm) and

θ = angle subtended by the zero and first order diffraction patterns.

Sarcomere length can be adjusted by moving the tension transducer back and forth on its micromanipulator.

The length-tension curve for skinned fibres has been determined by Moss (1979) on frog anterior tibial muscles. The results did not differ from those of Gordon et al. (1966) on intact fibres, with a tension plateau between 2.0-2.2 μm. Edman (1979) studied contraction velocity in tetanically stimulated fibres isolated from frog muscles, and found it to be independent of sarcomere length between 1.65-2.7 μm.

Dissection and mounting are done with the aid of binocular microscopes. A calibrated graticule in one eye-piece is used to measure fibre length and diameter in situ. Tension and length are recorded on a Tektronix 5113 dual beam storage oscilloscope. A 35 mm camera attachment is used to record the transients, and tension is continuously monitored on a Bryans 28000 chart recorder.

Dissection of Fibres

Fish are killed by a blow to the head and subsequently pithed. Small strips of red (slow) and white (fast) myotomal muscle are taken from the larger myotomes, pinned to small cork boards and kept in ice cold ringer. Bundles of 10-20 fibres are gently dissected from a myotome by cutting them free from the myosepta, and teasing them from the main fibre mass. Care is taken to touch only the ends of the fibres. Bundles are placed immediately in a shallow (2 mm deep) glass trough containing silicone oil at 0-5°C. A small drop of standard relaxing solution is injected into the oil around the fibres.

Fibre Attachment and Skinning

Single fast fibres, or small bundles of slow fibres, are teased from the main bundle. Because of difficulties encountered in mechanically skinning some species, chemical skinning was routinely adopted in all

experiments, as described below. The fibres are rapidly transferred between the tips of fine forceps, to the hooks of the apparatus. The ends are wrapped around the hooks and held in place by a drop of plexiglass/acetone glue, as shown in Figure 4:3. The fibre is then immersed in the first incubation solution. The thin covering of silicone fluid helps to prevent dehydration, and transference of the fibre is usually complete within 15-30 s. The first incubation solution contains normal relaxing solution (see below), with 1% Brij 58 (polyoxyethylene 20 cetyl ether) a non-ionic detergent. A 30 min. incubation makes the membrane completely permeable to external solutes (J. C. Kentish, personal communication). Diffusion of the relaxing solution components to the centre of the fibre has occurred in this time (e.g. Godt, 1974). The fibre is then transferred to relaxing solution without Brij 58 for > 3 min. Fibres are activated by immersion in activating solutions of varying free Ca^{2+} concentration. The exact protocol varies with the experiment, and will be described later.

Immediately after attachment, the sarcomere length is set at 2.3 μm, and the fibre length and diameter measured. After the first activation cycle sarcomere length may decrease to 2.1-2.2 μm at the centre of the preparation, possibly due to damage and subsequent extension at the ends. For this reason, sarcomere length was always checked after the first activation, and reset to 2.3 μm if necessary. Further shortening may occur, but this is generally < 0.1 μm.

Fibres or fibre bundles used in all experiments range from 50-200 μm in diameter.

SOLUTIONS

Chemical Supplies

All chemicals were analar grade reagents.

The following chemicals were obtained from Sigma Chemical Company (London):

EGTA (Ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetra-acetic acid)

Creatine kinase

Phosphocreatine

ATP

Silicone fluid MS 550

All other chemicals were obtained from BDH. BDH "extra pure" imidazole was used in all experiments.

Basic solutions

The basic relaxing solution has the following composition:

imidazole	10 mM pH 7.0 (at 8 or 0.5°C)
KCl	110 mM
$MgCl_2$	3 mM
EGTA	5 mM
Phosphocreatine	10 mM
ATP	2.5 mM

Creatine kinase in solid form is added just before each experiment to a final concentration of $\geq$ 20 $u.ml^{-1}$.

Imidazole, EGTA, phosphocreatine and ATP stock solutions are all set to pH 7.0 (at 8 or 0.5°C). The exact quantities of KOH or HCl added to set the pH is noted in all cases. An iterative computer programme (Perrin and Sayce, 1967), modified by White and Thorson (1972) is used to calculate the concentrations of the various ionic species in the solutions. The affinity constants used in the programme are listed in Appendix 1.

Activating solutions are made by the addition of $CaCl_2$ from 0-5 mM, to the basic relaxing solution. The concentrations of selected ionic

species in the relaxing and activating solutions are listed in Table 4:1.

Teleost and elasmobranch ringer compositions are given in Appendix 2.

Figure 4:1: Block diagram of the apparatus used in the study of the isometric and force-velocity relationships of skinned muscle fibres.

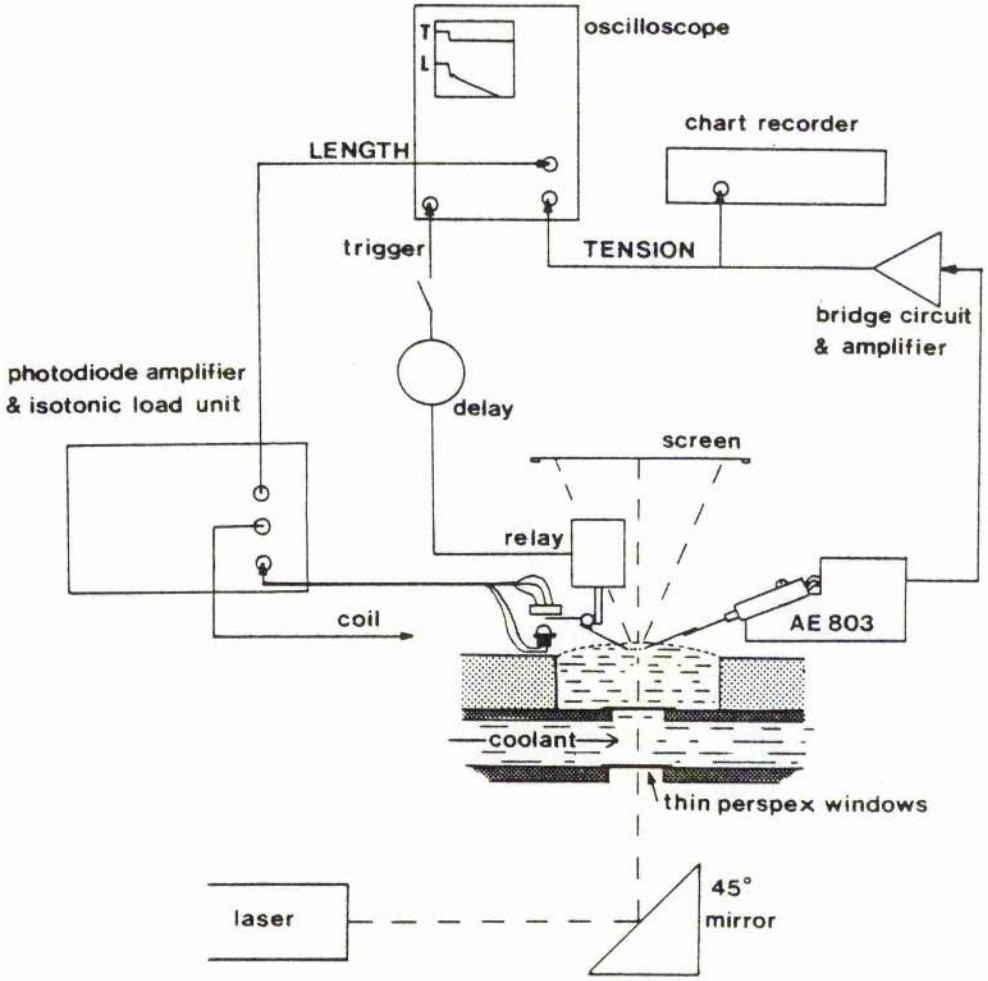

oscilloscope
T
L
chart recorder
LENGTH
trigger
TENSION
bridge circuit
& amplifier
photodiode amplifier
& isotonic load unit
delay
screen
relay
coil
AE 803
coolant
thin perspex windows
laser
45°
mirror

Figure 4:2: Details of the mechanical section of the apparatus.

B = incubation chamber; F = foil flag; H = glass hooks;

L = balsa wood lever; P = photodiode assembly;

S = stops.

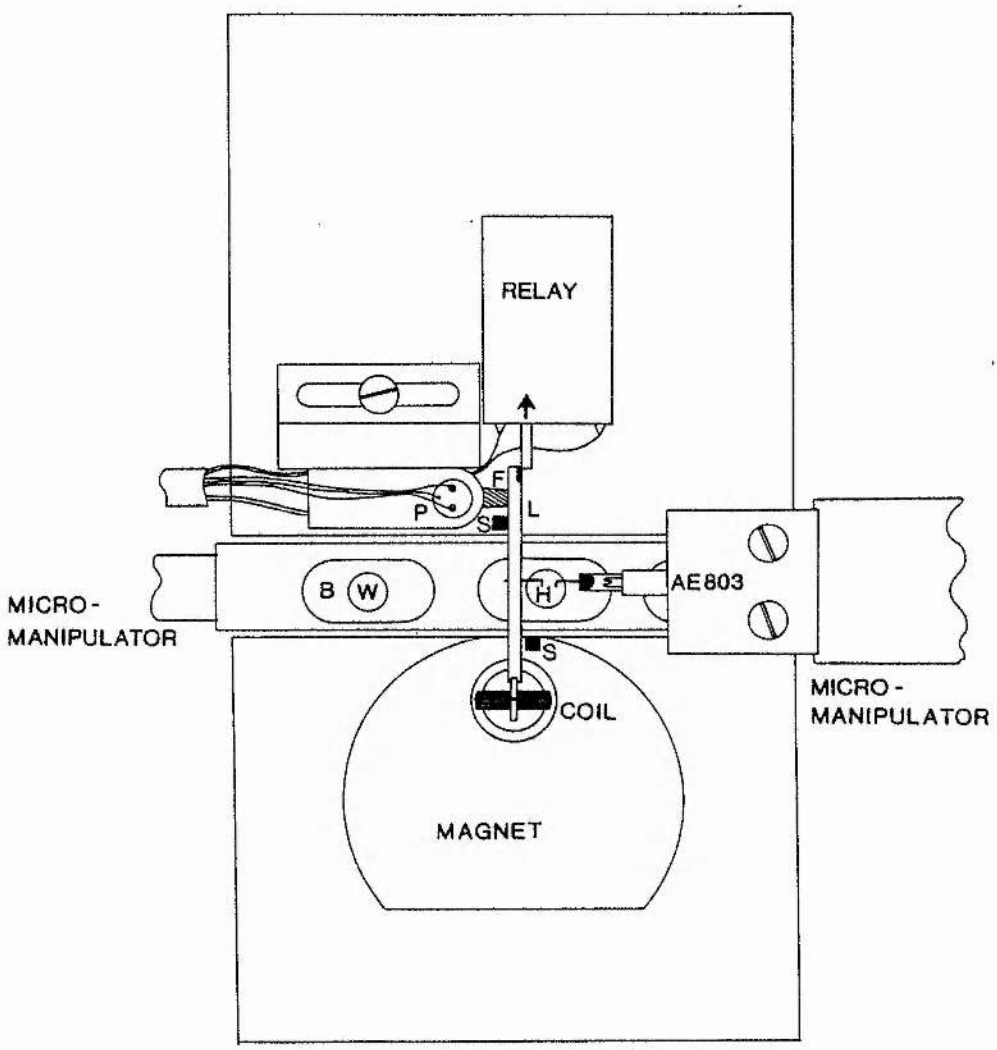
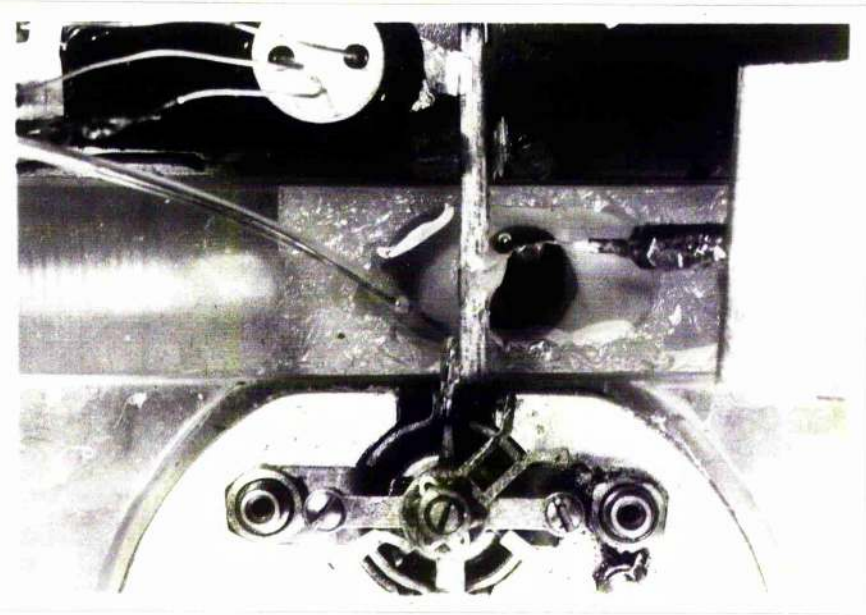
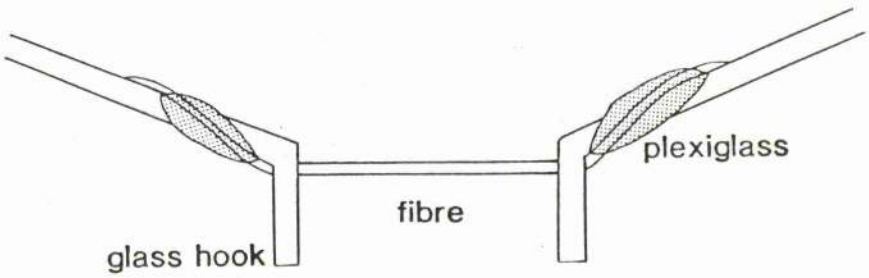

RELAY
F
P
L
S
B
W
H
AE803
MICRO-
MANIPULATOR
S
COIL
MAGNET
MICRO-
MANIPULATOR

Figure 4:3: (a) Detail of apparatus, with fibre attached.

(b) Schematic drawing to illustrate method of fibre attachment.



a



b

Table 4:1: The concentrations of selected ionic species in the relaxing and activating solutions.

Table 4:1

Solution	$[CaCl_2]$ (mM)	$[Ca^{2+}]_{free}$ (μM)	pCa	$[Mg^{2+}]_{free}$ (mM)	$[MgATP^{2-}]$ (mM)	Ionic strength (mM)
Relaxing	0	-	-	0.48	2.24	180.2
Activating	0.25	0.04	7.41	0.48	2.24	180.4
	0.50	0.08	7.08	0.48	2.24	180.6
	1.00	0.19	6.73	0.49	2.24	181.0
	1.25	0.25	6.60	0.49	2.24	181.4
	1.50	0.32	6.49	0.49	2.25	181.6
	1.75	0.40	6.39	0.50	2.25	181.9
	2.00	0.50	6.30	0.50	2.25	182.1
	2.50	0.75	6.13	0.51	2.25	182.6
	3.00	1.12	5.95	0.51	2.25	183.1
	3.50	1.75	5.76	0.52	2.25	183.6
	4.00	2.99	5.52	0.52	2.25	184.1
	4.50	6.58	5.18	0.53	2.25	185.0
	5.00	37.80	4.42	0.56	2.25	185.5

CHAPTER 5

THE ISOMETRIC TENSION PROPERTIES OF SKINNED FAST AND SLOW FIBRES ISOLATED FROM THE MYOTOMAL MUSCLE OF THE COD, *GADUS MORHUA*, AND THE DOGFISH, *SCYLIORHINUS CANICULA*

INTRODUCTION

In this chapter, studies on the steady isometric and pCa-tension characteristics of cod and dogfish myotomal muscles will be presented. The results will be discussed and compared with previous work.

The complex orientation of fibres within fish myotomes makes studies of their mechanical properties difficult. The few experiments performed on fish muscle have been restricted mainly to fin and jaw muscles. For example, Hidaka and Toida (1969) studied nerve stimulated and K^+ contractures in the pectoral muscles of the silver carp, but concerned themselves mainly with the biophysical membrane properties. The twitch/tetanus characteristics of fast and slow myotomal fibre bundles from the cod and the cuckoo ray, *Raia naevus* have been compared by Johnston (1980a). Flitney and Johnston (1979) studied the force-velocity characteristics of electrically stimulated fast and slow fibre bundles isolated from the adductor operculi muscles of *Tilapia*. Isometric tensions were 0.17 and 0.41 kg cm^{-2} for slow and fast fibres respectively (unpublished data). These values are very much lower than those obtained from intact amphibian and mammalian muscles (for review, see Prosser, 1973). It may be that this is a genuine phylogenetic difference, however the low tensions are more likely to be due to damage caused during dissection. The diffuse and numerous end plates on the fibres, and the large number of axons running between them, may cause considerable damage to the cell membranes during the separation of

small fibre bundles. Estimates of isometric tension will be low if many fibres are rendered inexcitable. This question can be resolved by using a skinned fibre preparation in which the normal excitation-contraction mechanism is uncoupled. Tension generation can be studied in isolation, and the muscle's full capacity for force production can be achieved.

METHODS

Fish

Cod, Gadus morhua, 45-60 cm in length were caught by commercial fishermen in the Firth of Forth. Dogfish, Scyliorhinus canicula, 50-65 cm in length were obtained from the Glasgow University Marine Laboratory, Millport, Great Cumbrae, in the Firth of Clyde. Fish were kept in the laboratory in recirculated, filtered sea water at 10 ± 1°C. Fish were killed by a blow to the head, and subsequently pithed. Small strips of red (slow) and white (fast) muscle were taken from the larger myotomes. In cod, this is immediately behind the head, and below the lateral line. Myotomes midway along the body were used from dogfish (Figure 5:1). Fibre dissection, mounting and skinning are described in Chapter 4.

Unless otherwise stated, experiments were carried out at 8°C.

Justification for the selection of the experimental conditions

The solution compositions (see Chapter 4, "Solutions") were determined in part from a series of preliminary experiments, described in the first Results section, and in part from information in the literature.

Thames et al. (1974) studied the effect of KCl concentration on contraction kinetics. Maximal isometric tension decreased continuously from 0 to 280 mM "added" KCl, and resting tension increased, after the first activation cycle, below 140 mM KCl. Gordon et al. (1973), and Julian and Moss (1981) also demonstrated a decrease in isometric tension

with increasing ionic strength. Gordon et al. (1973) comparing their data with those of others, obtained from intact fibres in hypertonic solutions suggest that a value of 140-170 mM for the ionic strength approximates physiological conditions. A value of 180 mM was chosen for the present experiments. In the solutions used, ionic strength varies from 180-185 mM. Using the data of Thames et al. (1974), this would produce a drop of < 2% in isometric tension, and a shift in the pCa-tension curve of < 0.04 pCa log units towards higher free Ca^{2+} concentration (Ashley and Moisescu, 1977). These effects are considered to be within the limits of resolution of the experiments.

Godt (1974) found that a decrease in Mg ATP^{2-} concentration from 2 mM (through 0.1 mM) to 20 μM shifted the pCa/tension curve to higher pCa, but did not affect maximum isometric tension. At very low $MgATP^{2-}$ concentration, resting tension increased dramatically, due to the formation of rigor complexes (Weber and Bremel, 1971; Bremel and Weber, 1972). Ashley and Moisescu (1977), working on barnacle, and Kerrick and Donaldson (1972) on frog skinned fibres, have shown that large changes in $MgATP^{2-}$ concentration in the millimolar range do not significantly alter the pCa/tension relationship.

The experiments of Ford and Podolsky (1972b) suggest that the concentration of free Mg^{2+} in the myofilament space is of the order of 10^{-3} M. Ashley and Moisescu (1977) demonstrated that a change from 1-5 mM free Mg^{2+} concentration shifted the curve 0.7 log units to higher free Ca^{2+} concentration without significantly affecting its steepness. In the solutions used in the present work, the small changes in free Mg^{2+} concentration ($\Delta[Mg^{2+}] = 0.08$ mM) with decreasing pCa will have a negligible effect on the steepness of the curve (< 0.017 log units over the total pCa range), and will not alter the relative positions of the curves from different fibre types.

The need for an ATP regenerating system has been emphasised by a

number of workers (e.g. Ashley and Moisescu, 1977; Godt, 1974). Ashley and Moisescu, with solutions containing 10 mM phosphocreatine and a $MgATP^{2-}$ concentration of 1-13 mM found that any increase in creatine kinase activity over 20 u. ml^{-1} produced no change in the isometric tension properties.

An EGTA concentration of 5 mM was chosen to avoid the delayed onset of force production and reduced rate of tension rise reported by Ford and Podolsky (1972a) at lower concentrations.

The pCa/tension relationship

Using the solutions described in Chapter 4, Table 4:1, the relationship between free Ca^{2+} concentration and steady isometric tension was investigated. Fibres were immersed in activating solutions until a steady tension developed before being returned to relaxing solution. The activating solutions were used in a random order, with maximally activating Ca^{2+} concentrations at intervals to monitor any decrease in P_0. A solution change could be effected in < 5 s. In some experiments, a fibre was transferred directly from one activating solution to another of higher or lower free Ca^{2+} concentration. Results obtained in this way did not differ from those obtained by relaxing the fibre after each activation.

The pCa-tension curves were linearised according to the Hill equation:

$$\log_{10}\left(\frac{P}{P_0-P}\right) = n \log_{10}[Ca^{2+}] + h \ ,$$

where n and h are constants. A straight line is obtained by plotting $\log_{10}(P / P_0-P)$ against pCa. When $P = 0.5P_0$, then $\log_{10}(P / P_0-P) = 0$. The Ca^{2+} concentration at this point is therefore that concentration required to give half maximal tension.

In all experiments, fibres were discarded when the diffraction

pattern became unclear, or P_0 fell below 75% of the maximal isometric tension of the first activation. The laser beam has a diameter of 1 mm, and thus samples upwards of 40% of the fibre length. As can be seen from Figure 5:2, the diffraction pattern is extremely sharp suggesting well registered sarcomeres. In those cod fast fibres which showed residual tensions, experiments were terminated when this rose to 20% P_0. Note, however, that the magnitude of this tension did not affect P_0 (see also Thames et al., 1974).

RESULTS

Effect of repeated activation-relaxation cycles on tension development

A number of experiments were performed to look at the effect of repeated activation-relaxation cycles on steady isometric tension (P) and resting tension. Maximal isometric tension (P_0) and the rate of rise of tension were not increased by raising the concentrations of $MgATP^{2-}$, creatine kinase or phosphocreatine.

Repeated activation-relaxation cycles could be performed at high and low Ca^{2+} concentrations with no increase in resting tension in all but cod fast fibres (e.g. 5.4, 5.9). In many cod fast fibre preparations, a residual tension in relaxing solution of 5-10% P_0 began to build up in successive activations after the first 2-3 cycles (Figure 5:3). In most experiments on slow fibres little or no decrease in P_0 was seen over 5-10 cycles. Fibres would occasionally undergo up to 15 cycles without a significant deterioration in P_0 or a loss in clarity of the diffraction pattern. P_0 in fast fibre preparations often remained constant over five successive cycles, but commonly decreased by 5-10%, most noticeably in cod (Figure 5:3). This phenomenon was prevalent only at Ca^{2+} concentrations greater than half maximal. The residual tensions observed in cod fast fibres were also seen only at high Ca^{2+}

concentrations. An increase in ionic strength, from 180 to 210 mM decreased P_0, but had no effect on the relative magnitude of the residual tension.

The pCa-tension relationship

The results obtained from a typical single cod slow fibre preparation are shown in Figure 5:4 to illustrate the experimental protocol. Pooled data on the pCa-tension relationship from 6 fast and 6 slow fibres from cod are given in Figure 5:5. In Figure 5:6 data from 8 fast and 5 slow dogfish fibres are presented. Each curve represents results from three fish. Tension is expressed relative to the maximum isometric tension obtained from each fibre. The threshold for tension generation in cod fibres is around pCa 7.2 (0.06 μM free Ca^{2+} concentration), and maximum tension is reached at pCa 5.18 (7 μM free Ca^{2+} concentration). The dogfish curves are somewhat steeper, maximum tension being reached at pCa 5.52 (3 μM free Ca^{2+} concentration). Linear transformations of the curves are shown in Figures 5:7 and 5:8. Half maximal pCa's were 6.08 and 6.42 for cod fast and slow muscle, and 6.41 and 6.50 for dogfish fast and slow fibres respectively.

The constant n in the Hill equation gives an estimate of the number of Ca^{2+} binding sites on the troponic C molecule (note, however, that other Ca^{2+} binding sites cannot be excluded, for example those on the myosin LC_2 (DTNB) light chain - see discussion). Gradients of 1.9 and 1.6 for cod fast and slow fibres suggest a minimum of two sites, with a higher degree of cooperativity in the fast muscle. The steeper dogfish curves give values of 3.5 and 3.2 for fast and slow muscles respectively. A minimum of four sites is therefore postulated, and again, the fast muscle exhibits the greater cooperativity. The shape of the pCa-tension curve is determined by the number of sites, and the degree of interaction between them. A single kinetic analysis such as

this cannot separate these factors, but can only give information about their combined effects. The situation is further complicated if the sites do not have identical km's for Ca^{2+}, and if the degree of interaction also varies between sites.

Steady isometric tensions

Force in skinned fibres has been shown to be proportional to cross sectional area (e.g. Hellam and Podolsky, 1969; Wise et al., 1971). Cross sectional area was calculated from the mean diameter, assuming circularity of the fibres. The small error which may arise from this assumption is minimised by taking the mean diameter, and by pooling results from a number of fibres. Maximum isometric tensions were 1.9 ± 0.12 (n = 11) and 0.85 ± 0.10 (n = 13) kg cm^{-2} for cod fast and slow, and 1.87 ± 0.09 (n = 28) and 0.84 ± 0.04 (n = 12) kg cm^{-2} for dogfish fast and slow fibres respectively. All values are mean ± S.E.

The effect of urea and TMAO on force production in dogfish

Dogfish muscle, in common with other elasmobranchs, contains high concentrations of osmoregulatory solutes, mainly in the form of urea and trimethylamine oxide (TMAO) (see, e.g., Prosser, 1973; Robertson, 1975). Fibres were precincubated, after skinning, for ten minutes in relaxing solutions containing 330 mM urea, 180 mM TMAO, or both urea and TMAO. Fibres were subsequently activated in solutions containing the same solute concentrations. The results are summarised in Table 5:1.

At 0.5°C, urea produced a drop in P_0 of 36%, and TMAO an increase of 7%. In the presence of both urea and TMAO, P_0 was lowered by 10%. At 8°C, P_0 was depressed by only 5% in the presence of urea and TMAO, possibly due to a reduced inhibition (22%) by urea.

The inhibition by urea did not appear to be fully reversible. Results from a representative preparation are shown in Figure 5:9.

DISCUSSION

Steady isometric tension

It is evident from these studies that maximum isometric tension is comparable to values found for other vertebrate skinned muscle fibres. For example, 1.5 and 1.7 kg cm^{-2} for frog semitendonosus (Gordon et al., 1973; Hellam and Podolsky, 1969, respectively) and 1.34 for rabbit psoas (Wise et al., 1971). The values for frog muscle are somewhat lower than those obtained from live preparations of 2-3 kg cm^{-2} (Ramsey and Street, 1940; Gordon et al., 1966). In contrast, the tensions produced by intact fish fibres (0.41 kg cm^{-2} for fast fibres, and 0.17 kg cm^{-2} for slow fibres; Flitney and Johnston, 1979) are very much lower than those generated by the skinned fibres of the present study. This may be caused by damage during dissection, due to the distributed nature of teleost innervation.

The lower tensions produced by skinned fibres, relative to live fibres, in other species may be due to differences between the intracellular environment of the intact cell and the bathing solutions used for skinned fibres. A mechanically skinned fibre swells in relaxing solution, the filament lattice distance increasing by around 15% (Matsubura and Elliot, 1972), and this has been proposed as a possible source of the discrepancies. April and Brandt (1973), however, found no effect on tension generation of changes in the interfilament spacing caused by the swelling of intact cells in hypertonic solutions. The swelling itself may lead to an overestimate of cross sectional area if it is not taken into account in the calculations.

The swelling in mechanically skinned fibres is caused, in part at least, by swelling of the sarcoplasmic reticulum (Taylor and Godt, 1976). Chemical skinning with triton X-100 does not cause swelling in mammalian cardiac trabeculae (J. C. Kentish - personal communication). Fibre

diameters in this study were measured in relaxing solution before and after treatment with Brij 58. No evidence for swelling was obtained. Chemical skinning with detergents disrupts both the sarcolemma and the S.R. (J. C. Kentish - personal communication), and this may account for the absence of swelling. As yet, it is not known if the tensions generated by chemically and mechanically skinned fibres are significantly different.

Maximum isometric tension in slow fibres was approximately half that of the fast fibres. This would be predicted qualitatively from ultrastructural studies on fish muscle. The fractional volume occupied by myofibrils varies from 60-86% in fast muscle, to 40-60% in slow (for review, see Johnston, 1980b). However, the difference is not fully explained by the relative fractional myofibrillar volume. Although no quantitative data are available for cod or dogfish, studies with other fish indicate that there are only around 50% more myofibrils in fast fibres than in slow. On this basis, it would appear that the tension generated per myofibril is greater in fast than in slow fibres.

Residual tension

The residual tensions observed in cod fast fibres after several activation cycles are not an unusual feature. They have been observed and studied by a number of workers. Unfortunately, no clear picture has arisen, due to differences in the temperature, ionic strength and experimental protocol used by the various groups. A brief summary of this work is therefore given.

Gordon et al. (1973) and Thames et al. (1974), working on frog semitendonosus fibres (mechanically skinned), demonstrated that both resting tension and maximum isometric tension were dependent upon ionic strength (μ). Both demonstrated that P_0 fell as ionic strength was increased from 90 mM to > 300 mM, and that at low ionic strength resting

tension increased. Both report that after contractions at low ionic strength, relaxation was incomplete.

(Note that Gordon et al. refer to ionic strength, Thames et al. to "added KCl". Using the data given by Thames et al., the ionic strength of relaxing/activating solutions with 50, 90 and 140 mM added KCl were calculated to be approximately 90, 140 and 190 mM respectively.)

Thames et al. found that at 5-7°C resting tension was not directly affected by ionic strength in relaxing solution, but that after a contraction at 90 mM added KCL, resting tension increased. The change was irreversible since a 30 min. soak in 140 mM added KCl relaxing solution had no effect on resting tension. However, working at 20-22°C Gordon et al. found that resting tension increased significantly when fibres were moved from relaxing solution at μ = 170 mM to μ = 90 mM (see Figure 1, Gordon et al., 1973). The incomplete relaxation observed after activation at μ = 90 mM could be reversed by mechanical perturbation. Are we looking at two different phenomena at 5 and 20°C? Using the same solutions later used by Thames et al., Hellam and Podolsky (1969) observed irreversible (contraction induced) residual tensions at 20°C and an ionic strength of 190 mM. If at an ionic strength of 190 mM (20°C) an irreversible change in resting tension occurs, and the change is ionic strength dependant, one would expect a similar irreversible change at 90 mM (20°C). The solutions used by the two groups differ only in that Thames et al. buffer with 10 mM imidazole, Gordon et al. with 10 mM Tris. Large incremental increases in resting tension are a common feature in the literature, e.g. in Donaldson and Kerrick (1975, Figures 1 and 2) at an ionic strength of 150 mM (20°C). Working at 1, 7 and 10°C, Julian and Moss (1981) observed a small contraction induced residual tension at μ = 90 mM, but not at 140 mM. However, the change was not completely irreversible (see their Figure 11 and text).

The data from cod may not be strictly comparable to those obtained

from frog. However, contraction induced residual tensions were seen in fast fibres at 8°C, and a further increase in ionic strength to 210 mM did not abolish these residual tensions. It is interesting to note that under the same conditions residual tensions were seen only once, in many experiments, with cod slow fibres. This was in a preparation which gave low, poorly reproducible tensions. This presumably reflects differences in the actin-myosin interaction between red and white fibres.

A possible mechanism for the effects of ionic strength on residual tension was proposed by Thames et al. (1974). Associated with the increased resting tension was a decrease in contraction velocity. They suggested that at low ionic strength, abnormal cross-bridge formation occurs which manifests itself as an increase in resting tension. This acts as an internal load during isotonic contraction, decreasing contraction velocity. Julian and Moss (1981), however, found no effect of ionic strength on contraction velocity in changing ionic strength from 90 to 140 mM, or from 140 to 180 mM, at 1, 7 and 10°C. Gulati and Podolsky (1978) also found contraction velocity to be independent of ionic strength at 1°C.

pCa-tension relationship

A broadly similar relationship between free Ca^{2+} concentration and isometric tension as found in the present study has been described for frog (Hellam and Podolsky, 1969), rabbit (Kerrick et al., 1976), and barnacle (Ashley and Moisescu, 1977). Although the results are not directly comparable due to differences in experimental solutions, they have a number of features in common. The curves are all sigmoidal, indicating multiple Ca^{2+} binding sites. The threshold for tension generation lies around pCa 7.0 (0.1 μM $[Ca^{2+}]$). The curve is steep, and maximum tension is achieved within 2 pCa units, at around 10 μM free $[Ca^{2+}]$.

In both cod and dogfish, the slow fibre curve is shifted to lower free [Ca^{2+}] relative to the fast fibres, indicating a lower k_m for Ca^{2+}. The data suggest that a minimum of 2 Ca^{2+} binding sites are involved in the activation of cod fast and slow fibres, and a minimum of four sites in dogfish fast and slow fibres. In both fish, the fast muscle exhibits a greater degree of cooperativity. These findings are very similar to those of Kerrick et al. (1976), who studied the pCa-tension relationship of fast and slow fibres isolated from rabbit muscle. The values quoted for n of 2.0 for adductor magnus (fast) and 1.7 for soleus (slow) are very close to those calculated for cod in the present study (1.9 fast and 1.6 slow fibres). The activation of contraction therefore involves a minimum of 2 Ca^{2+} binding sites.

Ca^{2+} binding sites have been identified on both the thick and thin filaments in skeletal muscle. Four sites were identified by Collins et al. (1973) on rabbit troponin C. Potter and Gergely (1975) identified two low affinity sites, and two high affinity sites, the latter also binding Mg^{2+}. It is generally accepted that the thin filament linked regulatory system acts through the troponin/tropomyosin complex, the first steps involving Ca^{2+} binding to troponin C (see Perry, 1979, for review). The Ca^{2+} sensitive regulation conferred by the thick filaments resides on the 2 DTNB (dithiodinitrobenzoic acid) or LC_2 light chains of the myosin heads (Kendrick-Jones et al., 1976). Many invertebrates posess only thick filament regulation (Szent-Gyorgyi, 1975). Most muscles, including those of the higher vertebrates, have both regulatory complexes, although the physiological importance of the LC_2 sites is in question. Some evidence does exist for a functional myosin linked regulation in vertebrates. Hazelgrove (1972) and Huxley (1972) demonstrated cross bridge movement during electrical stimulation in muscle stretched to a point where the actin and myosin filaments no longer overlapped. In a similarly stretched glycerinated preparation,

Chaplain and Gergs (1974) showed that the addition of Ca^{2+} moved cross bridges away from the myosin backbone. Lehman (1978) demonstrated a Ca^{2+} sensitive actomyosin ATPase activity in rabbit myofibrils treated in such a way as to remove the troponin/tropomyosin complex.

We therefore have a total of six sites which may be involved in the activation of contraction, four on troponin C, and one on each of the two myosin LC_2 light chains. Transient experiments with aequorin on intact fibres (Ashley and Moisescu, 1972), and later steady state experiments on skinned fibres (Ashley and Moisescu, 1977) suggest that only 2 Ca^{2+} ions are involved in the activation of barnacle muscle. This is in agreement with the concept of thick filament control only in many invertebrates put forward by Szent-Gyorgyi (1975). A kinetic analysis of isometric tension transients from frog skinned fibres led Moisescu (1976) to propose a minimum of 6 Ca^{2+} ions per functional unit in frog.

Biochemical studies on the Ca^{2+} regulatory proteins of fast and slow skeletal and cardiac muscles have revealed a number of structural differences. Three forms of rabbit troponin I have been identified on the basis of molecular weight and amino acid sequence (Syska et al., 1974). Slow skeletal and cardiac troponin C's have a similar primary structure in rabbit, but that of fast skeletal troponin C is very different (Perry, 1979). Perry (1979) also identified three forms of troponin T on the basis of molecular weight. Differences in the light chains of fast, slow and cardiac muscles are also well documented (e.g. Lowey and Risby, 1971; Sarkar et al., 1971; Weeds et al., 1975).

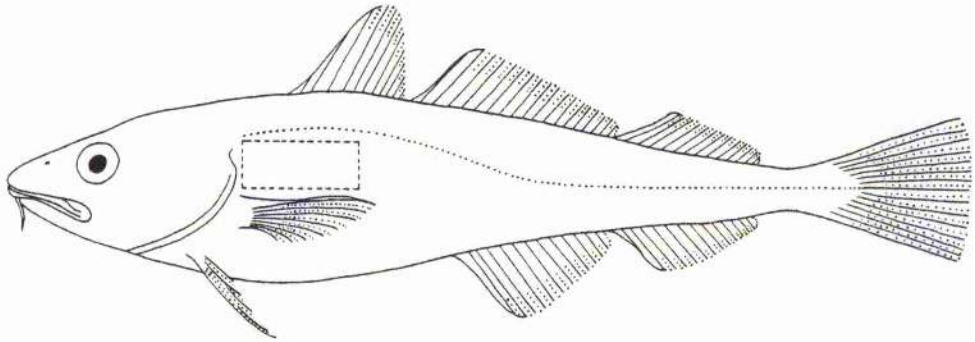
In conclusion, it would appear that the activation of both cod and dogfish myotomal muscle involves a minimum of 4 Ca^{2+} binding sites, located on the troponin C molecule. In addition, the 2 Ca^{2+} binding

sites on the myosin LC_2 light chains may be involved. The differences in k_m for Ca^{2+}, and the degree of cooperativity between sites, may be due to structural differences in any of the Ca^{2+} binding proteins, or the troponin T and troponin I molecules.

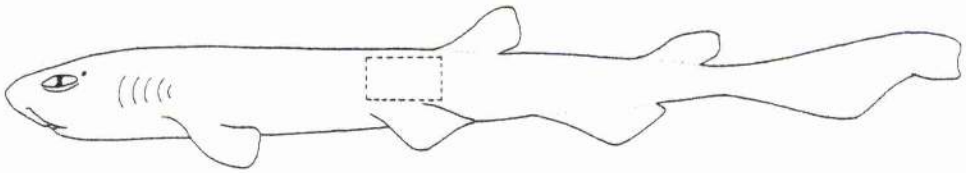
The effect of urea and TMAO on tension generation in the dogfish

Marine elasmobranchs contain high concentrations of urea and methylamine compounds, in an approximately 2:1 molar ratio, as osmotic agents, bringing intracellular osmolality to that of sea-water (Prosser, 1973). There is evidence to suggest that in the concentrations present the destabilising effect of urea on the cell proteins is counteracted by the methylamine compounds, made up mainly of trimethylamine oxide (TMAO) (Yancey and Somero, 1979). The concentrations of urea and TMAO used in the present study are those reported for the spiny dogfish, Squalus acanthus (Robertson, 1975). The effects of urea and TMAO on P_0 support this theory. At 8°C, the inhibitory effect of urea is almost totally abolished by the addition of TMAO. The absence of the minor methylamine compounds (e.g. betaine and sarcosine) normally present in the muscle may account for the small inhibitory effect still observed. At 0.5°C the inhibition by urea is enhanced. A temperature dependence of urea inhibition has previously been reported by Rajagopalan et al. (1961).

Figure 5:1: Sampling positions for the myotomal strips used to isolate skinned fibres



COD, Gadus morhua



DOGFISH, Scyliorhinus canicula

Figure 5:2: Laser diffraction pattern from a cod fast fibre. The spread in the zero order band is caused by direct transmission of light on either side of the fibre, since it is of a smaller diameter than the laser beam.

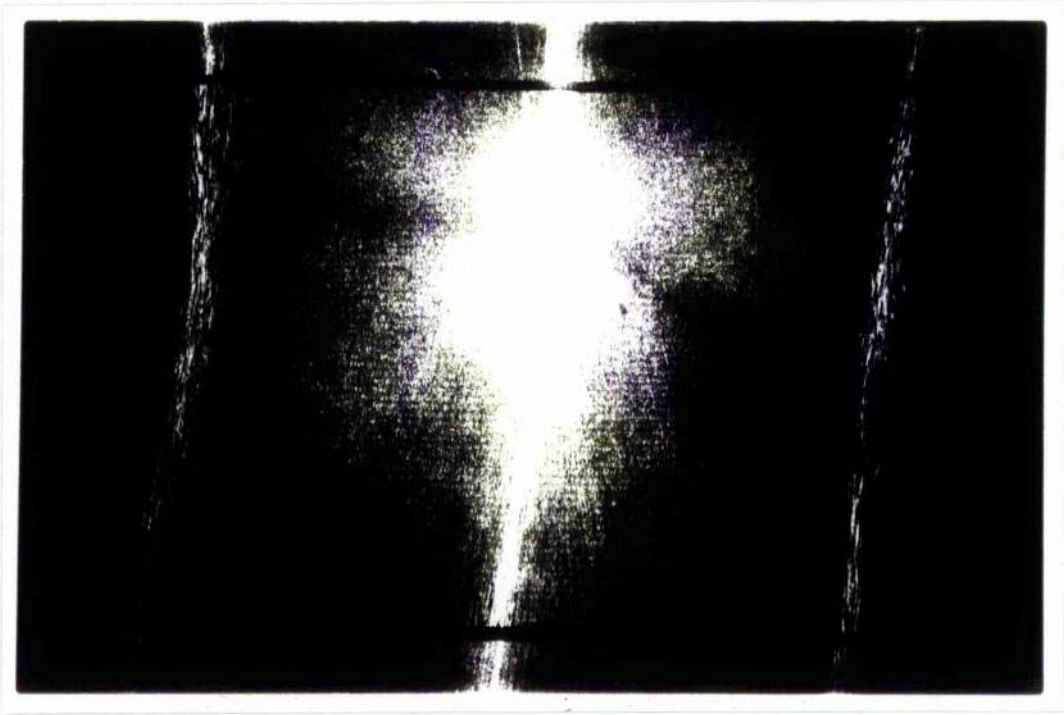


Figure 5:3: A series of successive maximal isometric activations on a fast cod fibre. Note the steady decline in P_0 and the rate of rise of tension, and the progressive increase in residual tension on transference to relaxing solutions. Arrows indicate changes to relaxing/activating solutions.

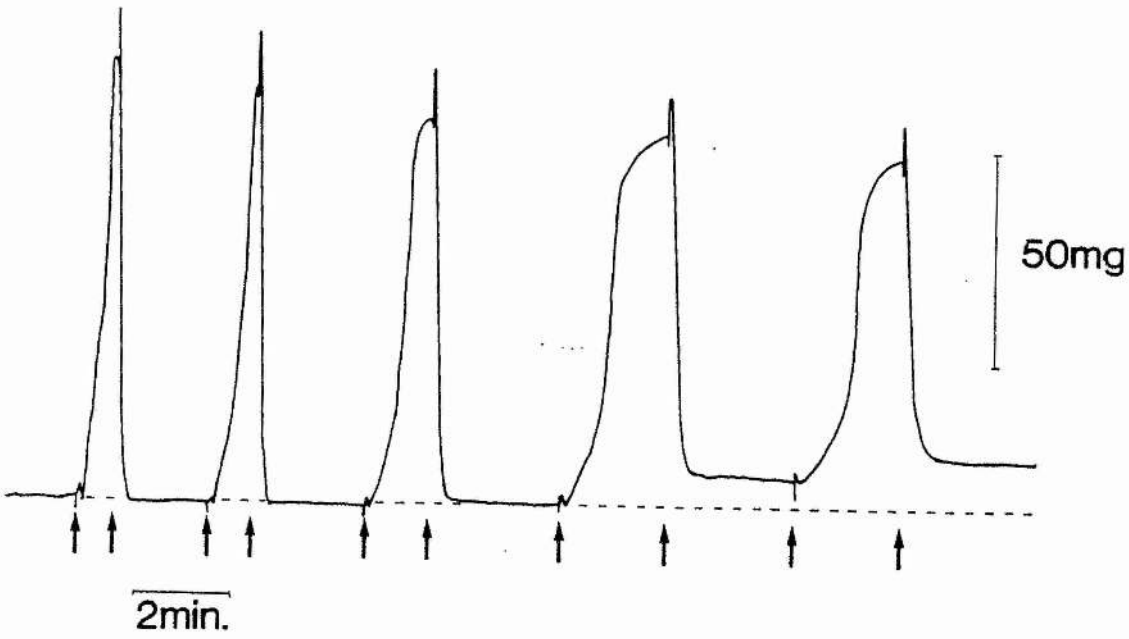
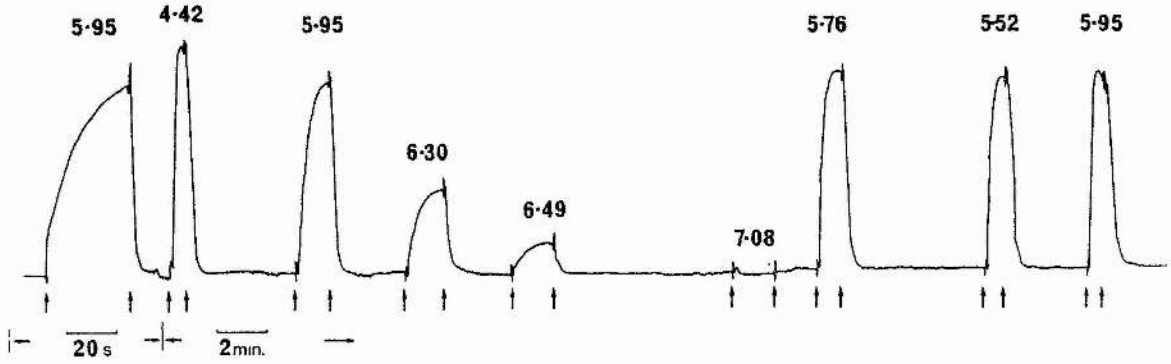

50mg
2min.

Figure 5:4: A series of isometric activations at different free $[Ca^{2+}]$ in a single cod slow fibre preparation. Arrows indicate changes between activating/relaxing solutions. The figures above the activation curves are the pCa values of the activating solutions.


5·95
4·42
5·95
6·30
6·49
7·08
5·76
5·52
5·95
20 s
2min.

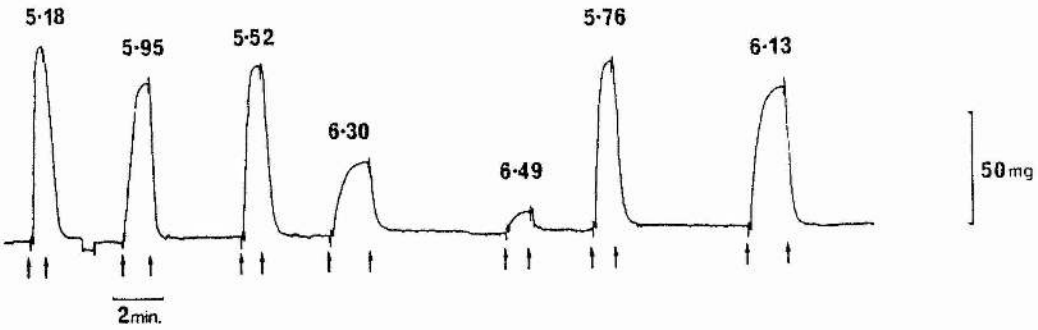

5·18
5·95
5·52
6·30
6·49
5·76
6·13
50mg
2min.

Figure 5:5: pCa-tension relationshipfor cod myotomal muscle. Open circles = fast fibres (n = 6); closed circles = slow fibres (n = 6). Numbers refer to the number of observations. The values are mean ± 1 S.E.

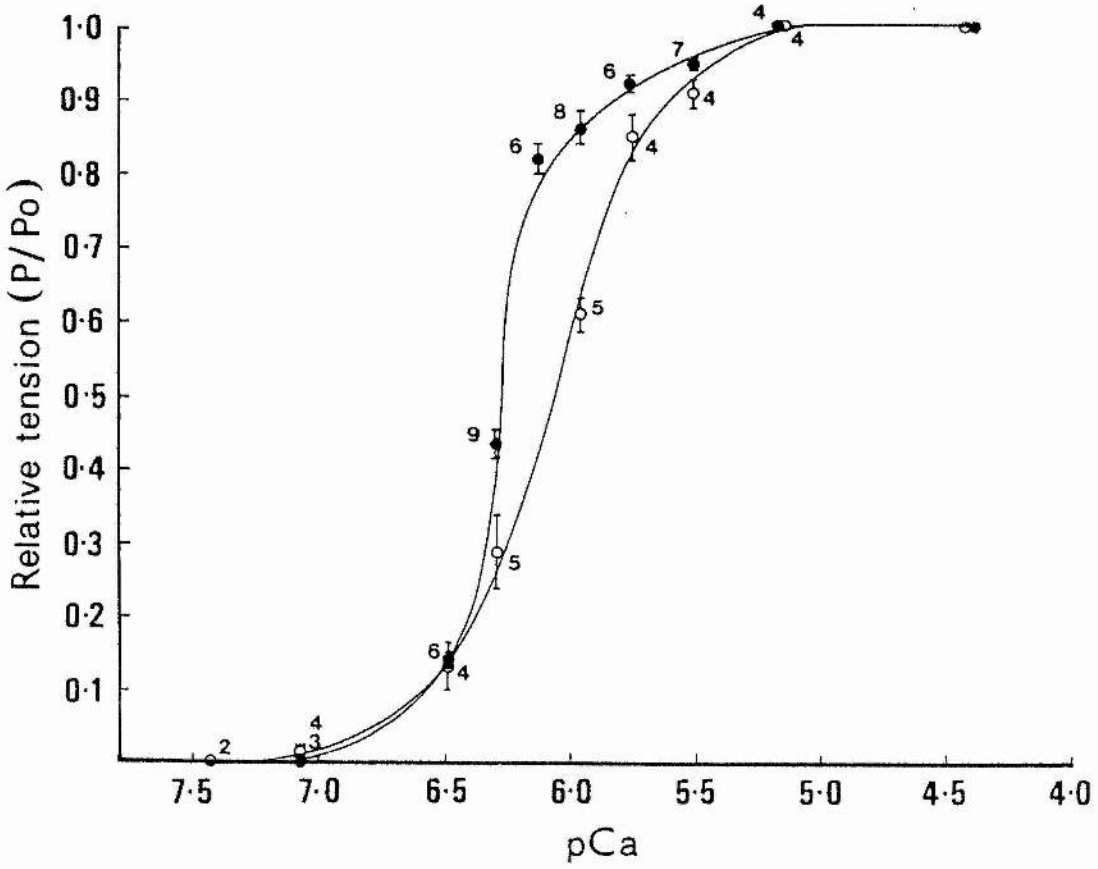

Relative tension (P/Po)
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
7·5
7·0
6·5
6·0
5·5
5·0
4·5
4·0
pCa

Figure 5:6: pCa-tension relationship for dogfish myotomal muscle. Open circles = fast fibres (n = 8); closed circles = slow fibres (n = 5). Numbers refer to the number of observations. The values are mean ± 1 S.E.

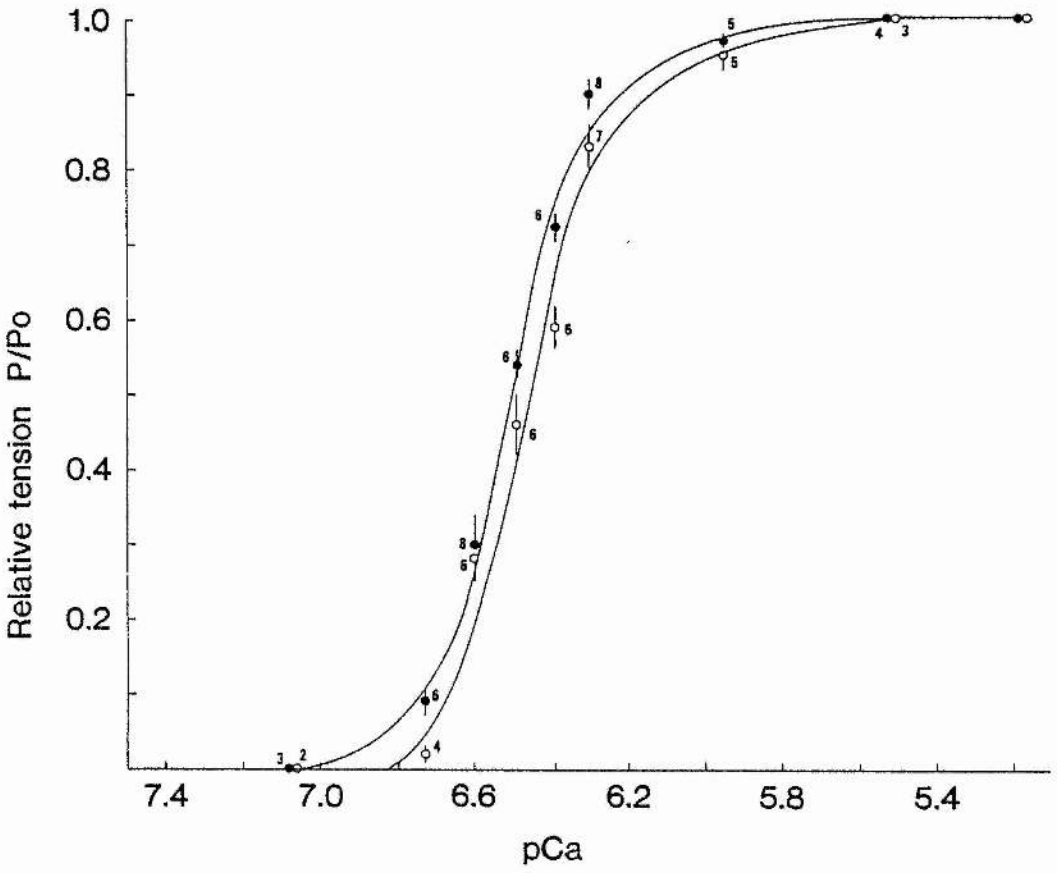

1.0
0.8
0.6
0.4
0.2
Relative tension P/Po
7.4
7.0
6.6
6.2
5.8
5.4
pCa

Figure 5:7: Hill plot of log $((P/P_0)/(1 - P/P_0))$ against pCa for cod fast (open circles; r = 0.99) and slow (closed circles; r = 0.98) fibres.

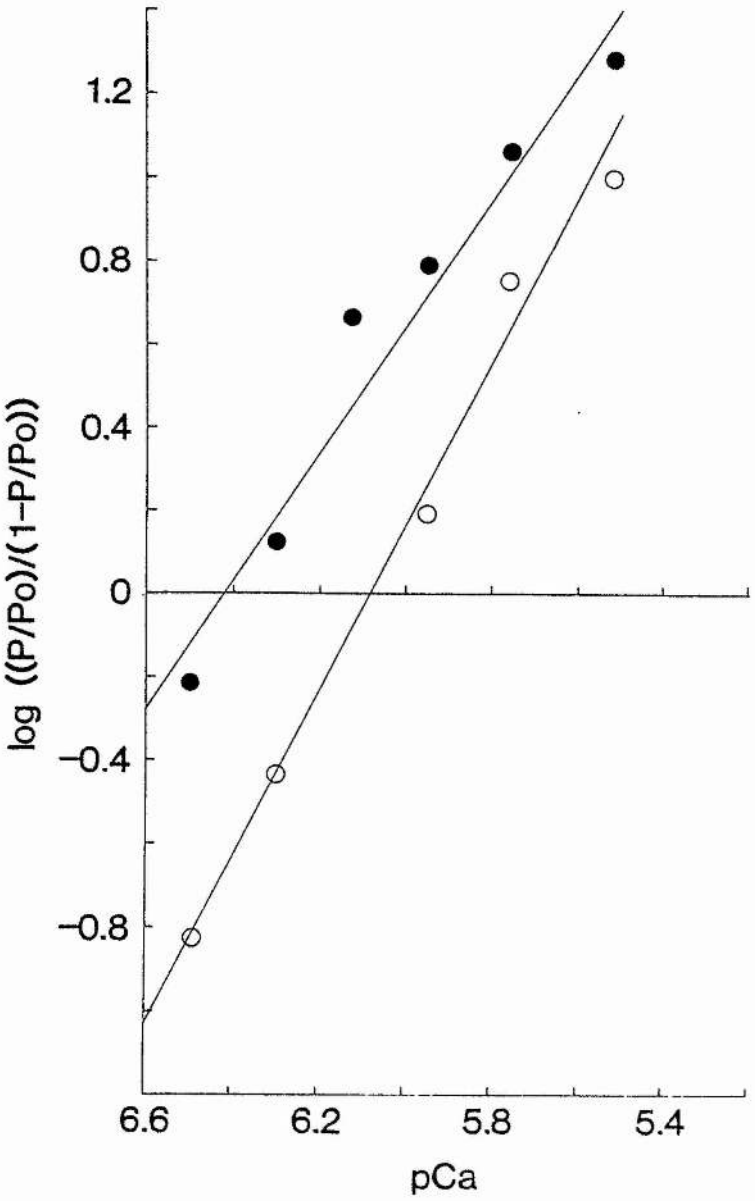

1.2
0.8
0.4
0
−0.4
−0.8
log ((P/Po)/(1−P/Po))
6.6
6.2
5.8
5.4
pCa

Figure 5:8: Hill plot of log $((P/P_0)/(1 - P/P_0))$ against pCa for dogfish fast (open circles; r = 0.94) and slow (closed circles; r = 0.97) fibres.

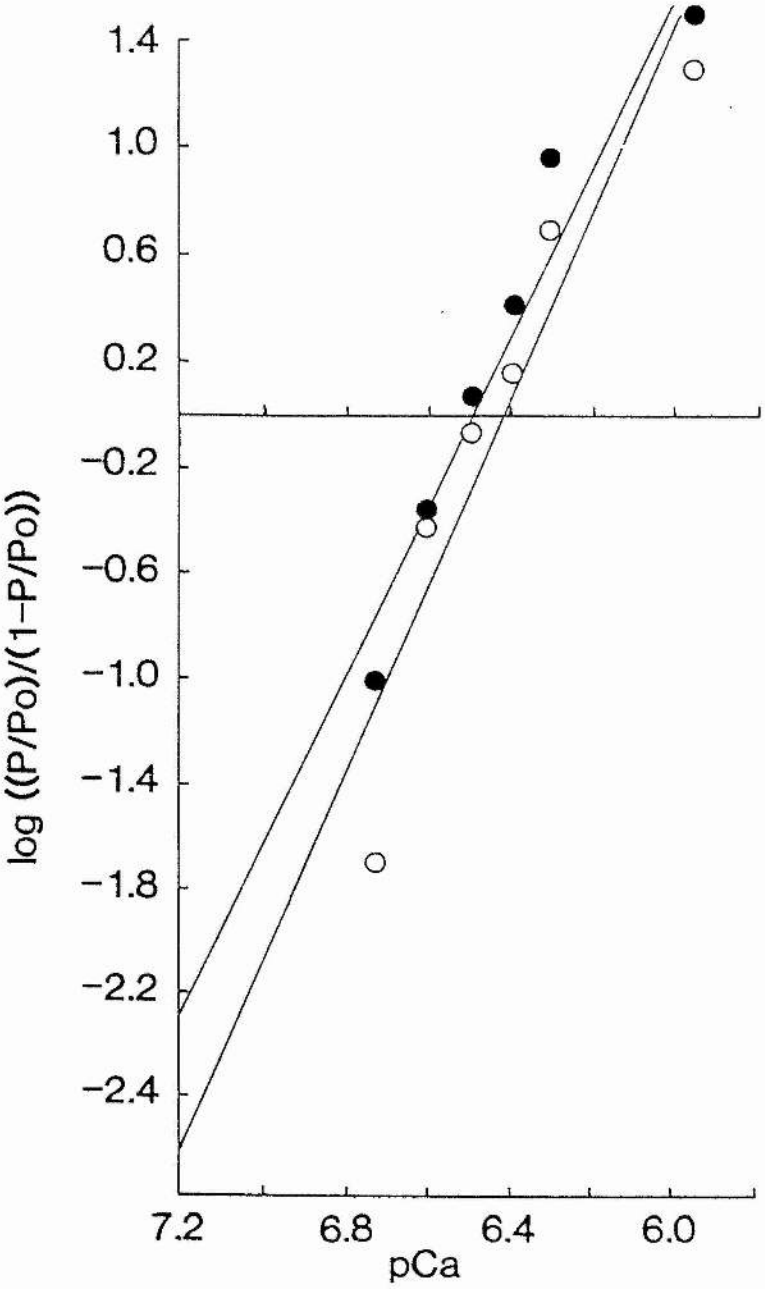

1.4
1.0
0.6
0.2
−0.2
−0.6
−1.0
−1.4
−1.8
−2.2
−2.4
log ((P/Po)/(1−P/Po))
7.2
6.8
6.4
6.0
pCa

Figure 5:9: The effect of osmoregulatory solutes on maximum isometric tension in dogfish fibres. Results from a typical slow fibre at 0.5°C. Time calibration = 2 min. during contraction cycles, 4 min. during incubations. Arrows indicate solution changes. A = activating solution; R = relaxing solution; U = + urea; T = + TMAO; UT = + urea and TMAO.

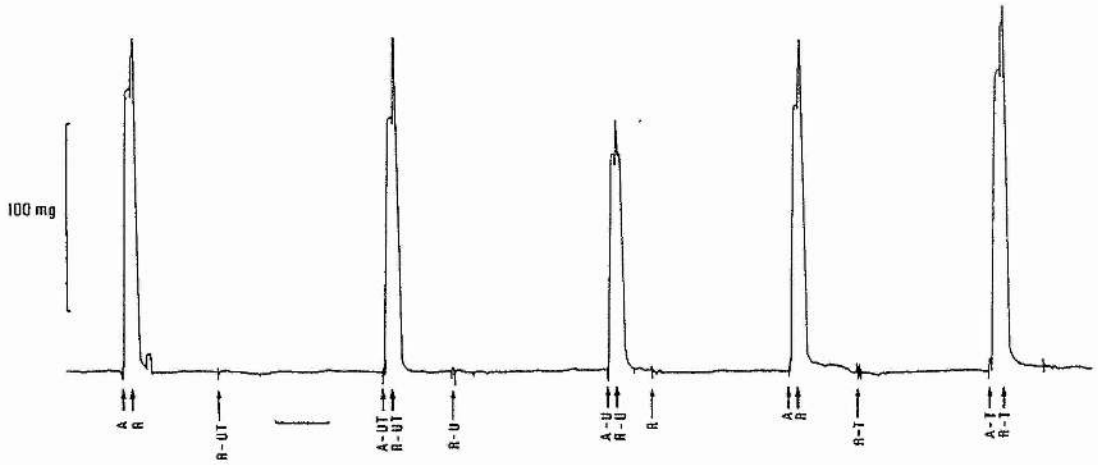

100 mg
A
R
R-UT
A-UT
R-UT
R-U
A-U
R-U
R
A
R
R-T
A-T
R-T

Table 5:1: The effect of physiological concentrations of osmoregulatory solutes on maximum tension in dogfish skinned fibres

Fibre type (Number of fibres)	Temperature	$\frac{P_{0(solute)}}{P_0} \times 100\%$		
		Urea	TMAO	Urea + TMAO
		(Number of observations)		
Fast (n = 4)	0.5°C	68.5 ± 2.29 (n = 6)	106.5 ± 2.51 (n = 4)	90.4 ± 0.95 (n = 6)
Slow (n = 3)	0.5°C	64.0 ± 2.51* (n = 3)	108.7 ± 2.85 (n = 3)	89.5 ± 1.50 (n = 3)
Slow (n = 6)	8°C	77.6 1.73* (n = 9)	104.0 1.29 (n = 4)	95.1 1.43 (n = 9)

* $p < 0.01$

CHAPTER 6

THE FORCE-VELOCITY CHARACTERISTICS OF SKINNED FAST AND SLOW FIBRES ISOLATED FROM THE MYOTOMAL MUSCLES OF THE COD, *GADUS MORHUA*, AND THE DOGFISH, *SCYLIORHINUS CANICULA*

INTRODUCTION

As noted in the last chapter, there have been few attempts to study the mechanical properties of fish muscle. The only previous study of the P-V characteristics is that of Flitney and Johnston (1979) on intact fibre bundles isolated from the adductor operculi muscles of *Tilapia mossambica*. Maximum contraction velocities at 18°C were 2.57 and 1.5 L s^{-1} for fast and slow fibres respectively.

The fast and slow fibres of all but the higher teleosts are somewhat comparable to those of amphibia. In both groups, the slow fibres are multiterminally innervated, and stimulation through the nerves elicits junction potentials (Stanfield, 1972). Note, however, that fish slow muscle is very much more aerobic than amphibian slow muscle. The mitochondrial fractional volume (25-35%, see Johnston, 1981b, for review) approaches that found in mammalian ventricular muscle. Fish and amphibian fast fibres are focally innervated, and stimulation, either through the nerve, or by depolarising pulses, elicits propagated action potentials (Hagiwara and Takahashi, 1967). Although elasmobranch fibres have only one end plate, they receive a dual innervation (Bone, 1972). The significance of this arrangement is unknown. The higher teleosts have evolved a pattern of innervation almost unique among the vertebrates. The polyneuronal innervation of these fish has been described in Chapters 1 and 2.

Few studies have been made of true slow fibres. Aidley (1965) studied isotonic contractions in frog rectus abdominus muscles during K^+ contractures. Floyd and Smith (1971) used a nerve-muscle preparation of the frog iliofibularis muscle in which the nerves to the fast muscle were selectively blocked. There are obvious disadvantages to studies involving whole muscles. Krugger et al. (1933) counted 36 slow fibres out of a total of 406 in the ilofibularis muscle of Rana temporaria, the rest being fast fibres. A value of 45 slow fibres out of 695 has been given for the same muscle in Xenopus laevis (Lannergren and Smith, 1966). Due to the smaller diameter of the slow fibres, they constitute only 2-3% of the cross sectional area of these muscles. In both mechanical and heat of shortening experiments, estimates must be made of the errors produced by the enormous bulk of surrounding fast fibres, acting both as a heat sink, and a passive resistence to shortening.

If these problems are to be overcome, experiments must be performed on isolated slow fibres. Only two such studies have been made prior to the present work. Costantin et al. (1966) looked at sarcomere shortening using high speed photography during the local application of droplets of Ca^{2+} solutions. Contraction velocity was ten times slower than in iliofibularis twitch fibres. Lannergren (1978, 1979) conducted a series of elegant experiments on intact single fibres isolated from an Anuran toad, Xenopus laevis. Force-velocity curves were obtained for iliofibularis twitch and slow fibres; maximum contraction velocities were 6.34 and 1.10 L s^{-1} respectively (22.5°C). A third class of fibre in the same muscle was later studied and found to have properties intermediate to the twitch and slow fibres. Maximum contraction velocity of these fibres was 2.17 L s^{-1}.

Comparisons between fast and slow mammalian twitch fibres are more numerous. Close (1964, 1965) and Luff (1981) investigated the P-V

relationship in whole soleus (predominantly slow fibres) and extensor digitorum longus (predominantly fast) muscles of the rat and mouse. Skinned fibre preparations from the same muscles of the guinea pig (Gulati, 1976) and rabbit (Julian et al., 1981) have also been studied. The relevance of these experiments to the present study will be discussed later in the chapter.

Because of the anatomical separation of the fast and slow fibre populations in fish myotomal muscle, it is relatively straightforward to isolate single fibres (for skinned preparations) from both types. These studies therefore represent not only the first such experiments on myotomal muscle, but add to our rather sparse knowledge of slow fibre mechanics.

PROTOCOL

Fast and slow myotomal fibres from both cod and dogfish were dissected and skinned as described in Chapters 4 and 5. Fibres were activated after the post-skinning incubation in relaxing solution. When tension reached a steady value, releases were given against a series of preset loads. In slow fibre preparations, up to ten releases could be given during a single maximal activation, without a significant decrease in tension. In a number of experiments, only one release was performed in each of 10-15 activation-relaxation cycles. The results obtained by the two methods did not differ. In experiments on fast fibres, only three to five releases were given in each cycle. Releases were usually given in a series of ascending or descending loads. This aided the rapid collection of data, decreasing the duration of the active state. Wherever possible, velocity measurements at low loads ($< 0.2\ P_0$) were repeated in the same or subsequent activation cycles. Results were not included in the final analysis if the contraction

velocity showed a consistent decrease at these low loads. Experiments were terminated when P_0 fell to < 70% P_0 of the first activation.

All the above experiments were performed at 8°C.

A number of experiments were performed at 2.5°C to study the effect of urea and TMAO on contraction velocity in dogfish fast fibres. Each fibre was given alternate 10 min. incubations in normal relaxing solution, and relaxing solution containing 330 mM urea and 180 mM TMAO. At the end of each incubation, contraction velocity was measured at low loads in maximally activating solutions containing the same osmoregulatory solute concentrations.

DATA ANALYSIS

Velocity transients were recorded from the oscilloscope with a 35 mm camera. Negatives were projected directly onto graph paper for analysis. A typical transient is shown in Figure 6:1 to demonstrate the method of analysis. Isometric tension, P_0, is dropped to a new value, P. Concomitant with this step force change, there is a rapid decrease in length, due to the series elastic component (SEC) of the muscle. The early events of the active contraction cycle are obscured to a varying extent by mechanical oscillation. Velocity measurements are taken over the second 50 ms interval after the onset of the step change in load. Some extrapolation through the oscillations was necessary at low loads.

Velocity in muscle lengths s^{-1} ($L\ s^{-1}$) was plotted against the relative tension P/P_0. Force velocity curves obtained in this way could be fitted to Hill's equation (1938):

$$(P+a)V = b(P_0-P) \ ,$$

where P = load, P_0 = maximal isometric tension, V = velocity, and a and

b are constants. Curves were linearised by plotting $((P_0-P)/P_0)/V$ against P/P_0. $1/V_{max}$ is given by the intercept with the y axis, a/P_0 by the intercept with the x axis, and 1/b by the gradient. Lines were fitted to the force-velocity curves by computer, using the constants a and b derived from the above analysis.

It has been shown that at high loads the experimental points on a force-velocity curve deviate markedly from the rectangular hyperbola predicted by Hill's equation (e.g., Hill, 1970; Edman, 1976, Lannergren, 1978), and points beyond 0.6-0.8 P_0 are usually omitted from the linear analysis. For this reason, velocity at high loads was not studied in the present work.

RESULTS

Results from a typical experiment (from cod slow fibres) is shown in Figure 6:2 to illustrate the experimental protocol. Both dogfish fibre types, and cod slow fibres, were very robust at all $[Ca^{2+}]$ concentrations (see Chapter 5). It was, however, difficult to obtain more than two or three releases from maximally activated cod fibres before they showed visible signs of non-uniformity and a marked deterioration in the clarity of the diffraction pattern.

Typical isotonic releases from cod fast and slow fibres at maximal and submaximal $[Ca^{2+}]$ are shown in Figure 6:3. Releases from maximally activated dogfish fast and slow fibres are given in Figure 6:4. A noticeable feature of all velocity transients was their departure from linearity. Transients from maximally activated fast fibres (and dogfish slow fibres) resemble those from previous studies on skinned fast fibres from frog (e.g., Podolsky and Teichholtz, 1970; Thames et al., 1974), in that deviations from linearity at low loads were small (velocity decreased < 25% in 250 ms). In all fibre types, the transients became

increasingly more curved at higher loads.

Velocity in cod slow fibres decreased rapidly initially, before slowing towards a uniform velocity, producing transients very similar to those reported by Aidley (1965) and Floyd and Smith (1971) from the slow fibres of frog rectus abdominus muscle.

Transients from half maximally activated cod fast fibres showed a greater departure from linearity than maximally activated fibres (Figure 6:3). The poor viability of cod fast fibres did not allow this effect to be studied in detail. In contrast, a number of releases at maximal and submaximal [Ca^{2+}] could be obtained from the same fibre in dogfish preparations. The effect of Ca^{2+} on the shape of the velocity transient could therefore be reliably quantified, and this phenomenon will be discussed in the next chapter. Due to the poor viability of cod fast fibres, and the curved nature and interfibre variability in slow fibres, no consistent differences were found in shortening velocity for sub- and maximally activated fibres. The data were therefore combined.

Force velocity curves from cod fast fibres are shown in Figure 6:5 (pCa 5.18 - 2 fish, 17 fibres; pCa 6.13 - 3 fish, 11 fibres), and from slow fibres in Figure 6:6 (pCa 5.18 - 3 fish, 7 fibres; pCa 6.30 - 3 fish, 6 fibres). V_{max}, as determined from the linear form of the Hill equation was 0.53 L s^{-1} for slow fibres; a = 0.28 P_0; b = 0.21 L s^{-1}. V_{max} = 1.02 L s^{-1} for fast fibres; a = 0.21 P_0; b = 0.21 L s^{-1}.

Force-velocity curves from maximally activated dogfish fast and slow fibres are shown in Figure 6:7 (3 animals, 5 slow preparations, 7 fast fibres). V_{max} for slow fibres was 0.68 L s^{-1}; a = 0.19 P_0; b = 0.13. Fast fibre V_{max} = 2.34 L s^{-1}; a = 0.06; b = 0.14.

The effects of urea + TMAO on contraction velocity in dogfish fast fibres are summarised in Table 6:3. It was concluded that urea + TMAO has no significant effect on velocity.

DISCUSSION

Maximum Contraction Velocity

Values of V_{max} and a/P_0 obtained in the present study, and those taken from the literature are summarised in Tables 6:1 and 6:2 respectively. V_{max} for cod and dogfish fast fibres is two to three times greater than in slow fibres. Flitney and Johnston (1979) report an approximately two-fold difference in Tilapia. This is in marked contrast to the values found for amphibian muscles, where a six to ten-fold difference has been reported (Costantin et al., 1966; Lannergren, 1978). The results obtained from fish are in fact more similar to those from mammalian fast and slow twitch fibres. As can be seen in Table 6:2, mammalian fast fibre V_{max}'s are two to three times greater than slow. Note that the table is not comprehensive, merely representative of the data in the literature.

It is thought that amphibian slow fibres are responsible only for maintaining posture, and therefore have no requirement for a high contraction velocity. However, the slow fibres of fish play an active role in locomotion. At slow and intermediate swimming speeds in elasmobranchs and other primitive fish, it is the slow fibre population alone which is active (Bone, 1966; Bone et al., 1978). In function, therefore, they have a role more analogous to the slow twitch fibres of mammalian skeletal muscle. This may be reflected in the small differences in V_{max} seen between fast and slow muscle.

The Force-Velocity Relationship

The degree of curvature of the force-velocity relationship is determined by the value of a/P_0. As a/P_0 decreases, the relationship becomes more curved. The values obtained for a/P_0 in the present study (Table 6:1) lie within the range found for other vertebrate muscles, listed in Table 6:2.

The degree of curvature of the force-velocity curve is thought to have functional significance. Hill (1938) suggested that the exact shape was connected with the heat produced, and that the rate of heat production (h) could be described by:

$$h = av + f(t) ,$$

where f(t) is a rate of heat production which falls during a tetanus to reach a steady value equal to a.b. From this, it follows that as a/P_0 decreases the ratio of work rate (P.v) to (work + heat) increases.

Woledge (1968) tested those predictions by studying the thermal and mechanical properties of tortoise rectus femoris muscle ($a/P_0 = 0.07$ at 0^oC), and comparing his results with those of Hill (1938) on frog sartorius ($a/P_0 = 0.26$ at 0^oC). Woledge concluded that tortoise muscle has a work rate to (work + heat) ratio 70% greater than that of frog muscle. He also found the efficiency of the whole cycle of contraction and recovery to be 70% greater. Some evidence is presented to suggest that tortoise muscle can obtain a greater amount of work per mole of phosphocreatine than frog muscle. Although the exact quantitative relations are not known, Woledge suggested that a more curved P-V relationship is associated with a more efficient conversion of free energy into work.

It is thought that as velocity increases the average life time of an attached cross bridge decreases, i.e. the number of cross bridges attached at a given time decreases. Cross bridges will also be moved toward the low tension end of their individual length-tension curves. As a result of these two factors, tension falls as velocity increases. The rate of cross bridge turnover will also increase as velocity increases, explaining the increased energy release during shortening. Inefficiencies will arise if cross bridges remain attached after passing into configurations where they oppose motion, and also if cross bridges

attach and detach at intermediate points in their length-tension curves. Woledge (1968) puts forward a number of mechanisms which would decrease these inefficiencies, and shows that they all tend to increase the curvature of the force-velocity relationship.

In any muscle, there must be a compromise between isotonic efficiency and power output, since an increased curvature of the P-V relationship will lead to a decrease in force at a given velocity. From a study of Tables 6:1 and 6:2, it can be seen that with the exception of dogfish fast muscle, all the fish fibre types investigated have similar values of a/P_0, within the range given for amphibian and mammalian fast muscles, suggesting an adaptation towards high power output. It is interesting to note that in higher teleosts such as the cod there is good evidence to suggest that fast fibres are recruited at intermediate sustainable swimming speeds, in addition to providing power for burst swimming (Johnston et al., 1977; Bone et al., 1978; Johnston and Moon, 1980b). This similarity of function may be reflected in small differences seen in a/P_0 in cod fast and slow fibres.

The low a/P_0 obtained for dogfish slow fibres cannot be explained on the basis of our present knowledge. If this question is to be resolved, a better understanding of both the mechanics and energetics of contraction during swimming, and in many more species, is required. Suggestions for further work will be considered in Chapter 8.

The Shape of the Isotonic Velocity Transient

At the end of the rapid shortening of the SEC, low amplitude, damped oscillations were superimposed on the active shortening phase of the release. The frequency of these oscillations is dependent upon the inertia and compliance of the lever system (which ideally are negligible), muscle compliance, and the load on the muscle. In common with previous measurements of skinned fibres, velocity over a similar time scale from

amphibian (e.g., Podolsky and Teichholtz, 1970; Julian, 1971; Thames et al., 1974) and mammalian (e.g., Wise et al., 1971; Gulati, 1976) muscles transients from maximally active dogfish fast and slow, and cod fast fibres, were approximately linear. Deviation from linearity was greater at high loads.

In submaximally activated cod fast fibres, the curvature of the transients increased. This Ca^{2+}-dependent inactivation was not studied in depth in cod because of the poor viability of the preparation at high $[Ca^{2+}]$. The effect has been studied in greater detail in dogfish, and will be discussed in the next chapter.

A striking feature of the cod slow fibre transients was the rapid decrease in velocity during a release, producing very curved transients. A similar phenomenon has been described in all three previous studies on slow fibres by Aidley (1965), Floyd and Smith (1971), and Lannergren (1978) (see Introduction). This "inactivation" was studied by Lannergren (1978) in intact single fibres during K^+ contracture. The degree of curvature was greater when the $[K^+]$ used to cause contracture was increased from 45 mM to 75 mM. Lannergren suggests that the rates of cross bridge attachment and detachment may be influenced by external $[K^+]$, possibly via an effect on internal free $[Ca^{2+}]$. The phenomenon may therefore be a more critically length dependent form of the Ca^{2+} and length dependent process described in the next chapter.

Figure 6:1: An isotonic release to illustrate the method of transient analysis. SEC = series elastic component. Tension is expressed as P/P_0. Mean velocity over the second 50 ms interval after the onset of release (a/b) is measured.

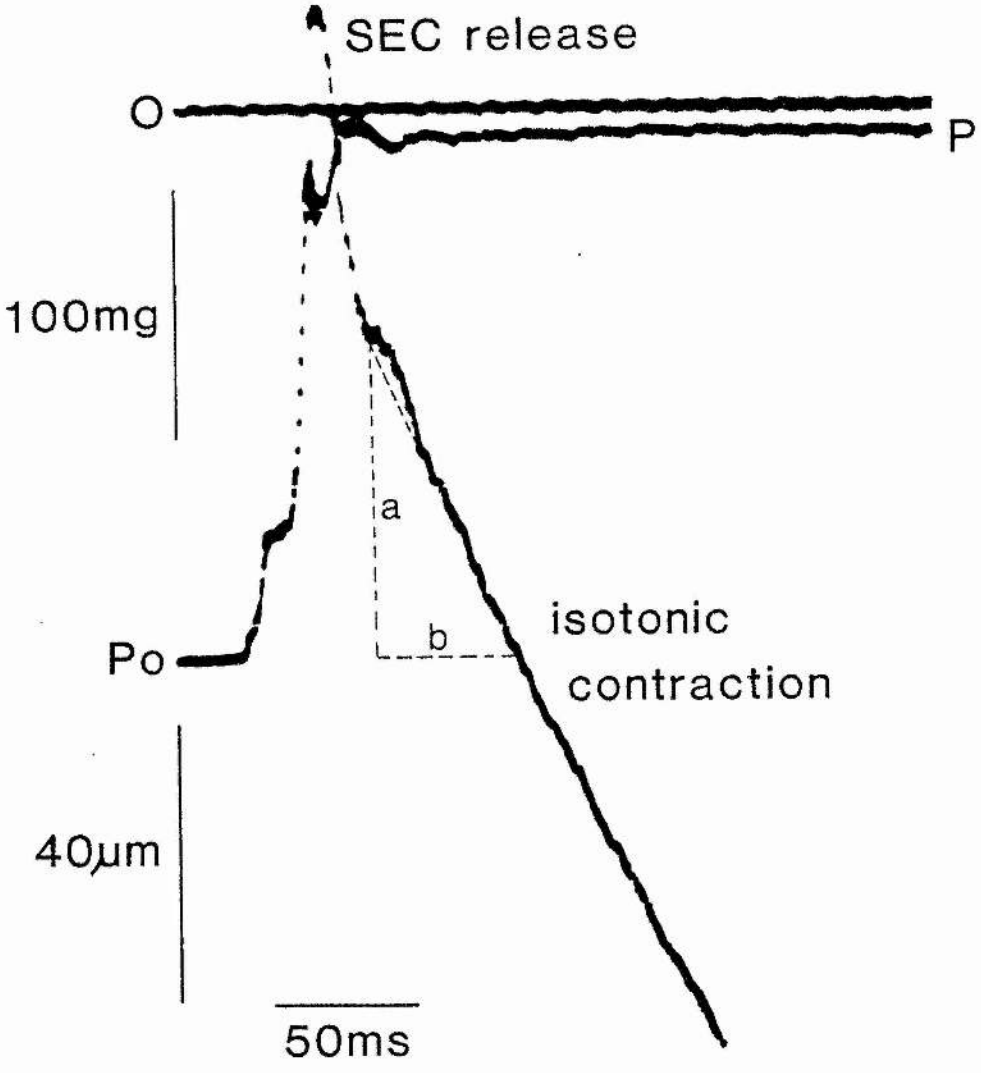
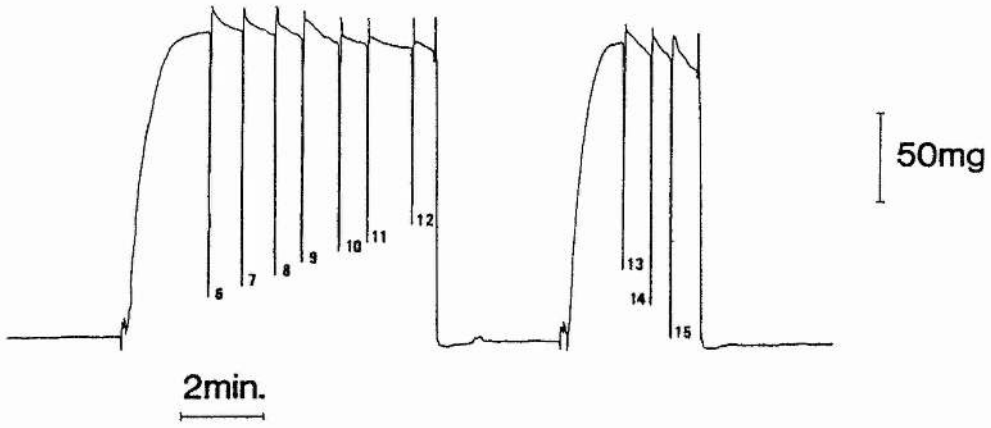

SEC release
O
P
100mg
a
b
Po
isotonic
contraction
40μm
50ms

Figure 6:2: A typical experiment (from cod slow fibres) to illustrate the experimental protocol for P-V determinations.

Top: Isometric record of second and third activations in a cycle of three. Numbers refer to the data points given below.

Bottom: Force-velocity curve from single slow fibre preparation. P/P_0 = relative tension (load/maximum isometric tension). Velocity is expressed in fibre lengths s^{-1}. Numbers against data points refer to the order in which they were derived.


50mg
2min.
5
7
8
9
10
11
12
13
14
15

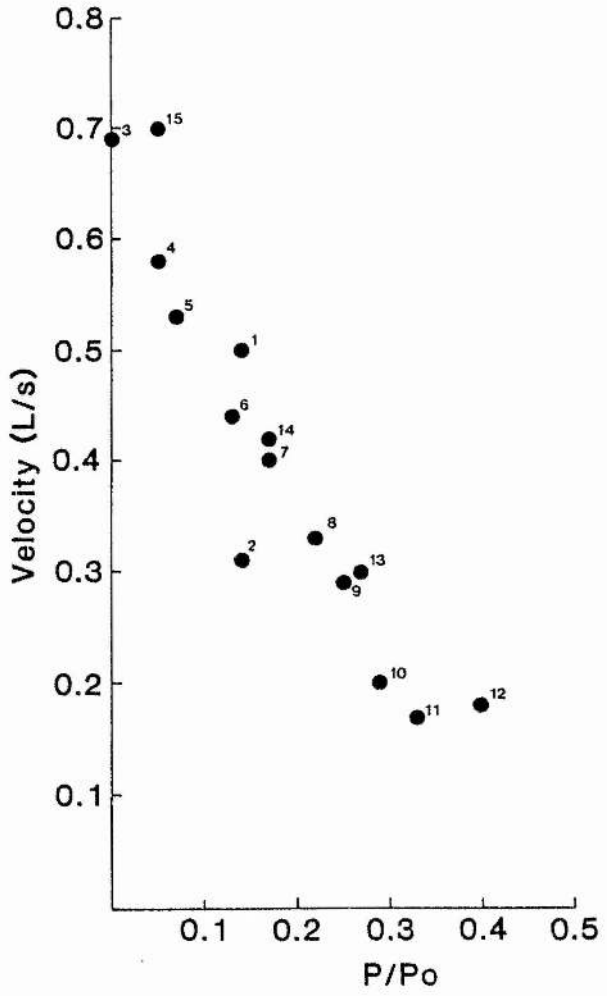
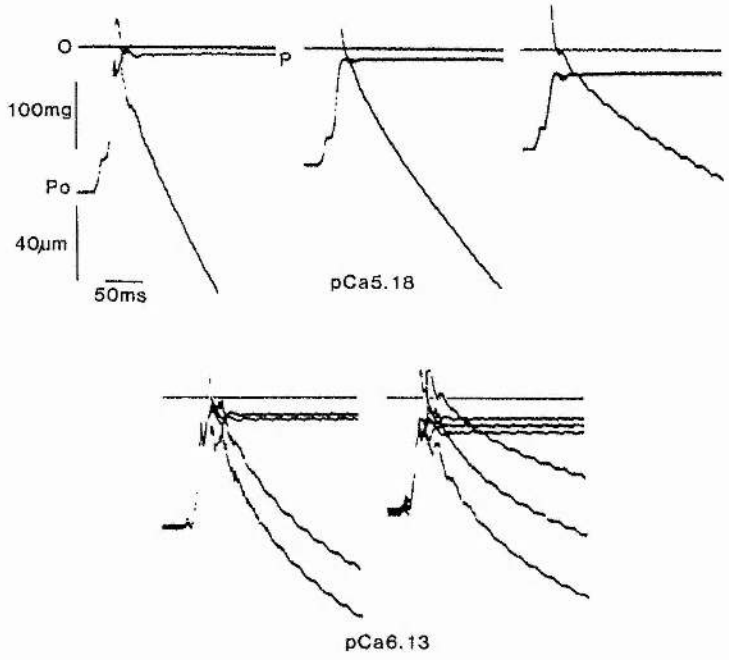

Velocity (L/s)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.1
0.2
0.3
0.4
0.5
P/Po
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 6:3: Isotonic releases from cod fast and slow fibres. Notation as in Figure 6:1. 8°C.

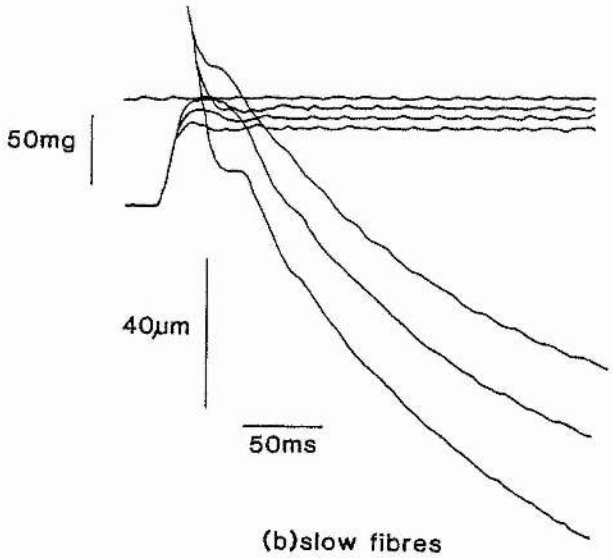
(a) Upper: fast fibres at maximal (pCa 5.18) free $[Ca^{2+}]$. Transients from three fibres.

Lower: half maximally (pCa 6.13) activated fast fibre transients from a single preparation. Note the greater degree of transient curvature at low $[Ca^{2+}]$.

(b) Slow fibres. Superimposed, retraced transients from a single preparation at pCa 5.18. No differences in transient shape at high and low $[Ca^{2+}]$ could be resolved, possibly due to the large inter-fibre variation observed. 8°C.



(a)fast fibres

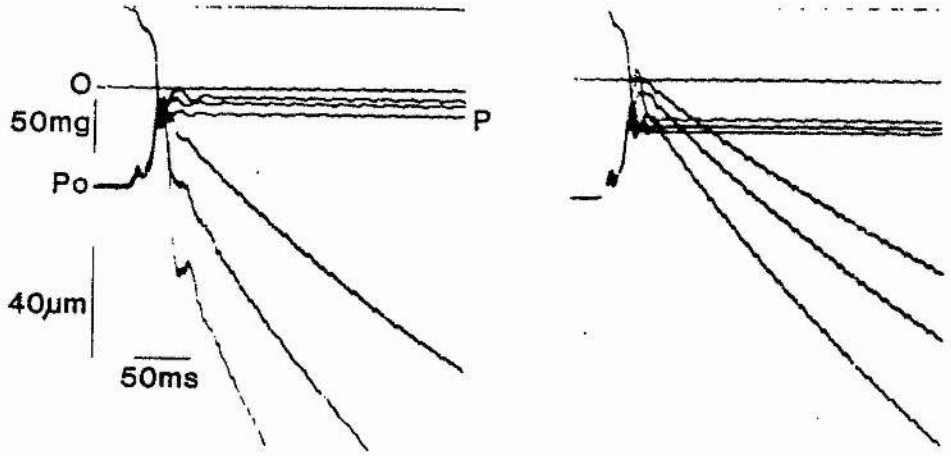


(b)slow fibres

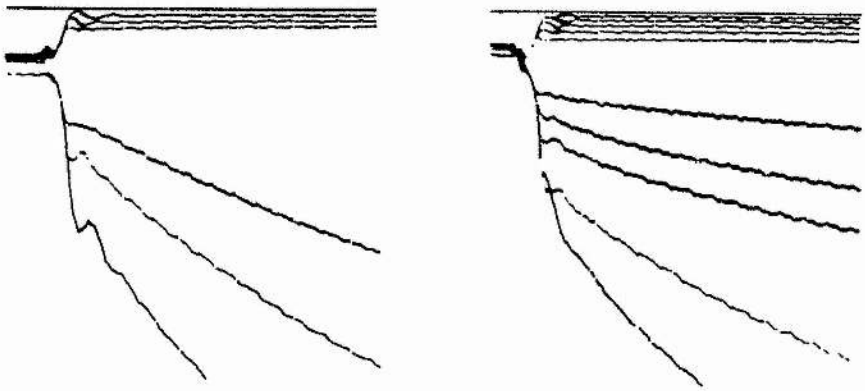
Figure 6:4: Isotonic releases from dogfish fast and slow fibres (maximally activated, pCa 5.52). Notation as in Figure 6:1. 8°C.

(a) transients from two fast fibres;

(b) transients from one slow fibre preparation.



(a) fast fibres



(b) slow fibres

Figure 6:5: Force velocity curves from cod fast fibres. Closed circles = half maximally activated (pCa 6.13), 3 fish, 11 fibres. Horizontal bars indicate range of data, vertical bars ± 1 S.E., and numbers = number of observations. Solid line computed from constants a and b. Open circles = data from maximally activated fibres (pCa 5.18), 2 fish, 17 fibres. Experiments performed at 8°C.

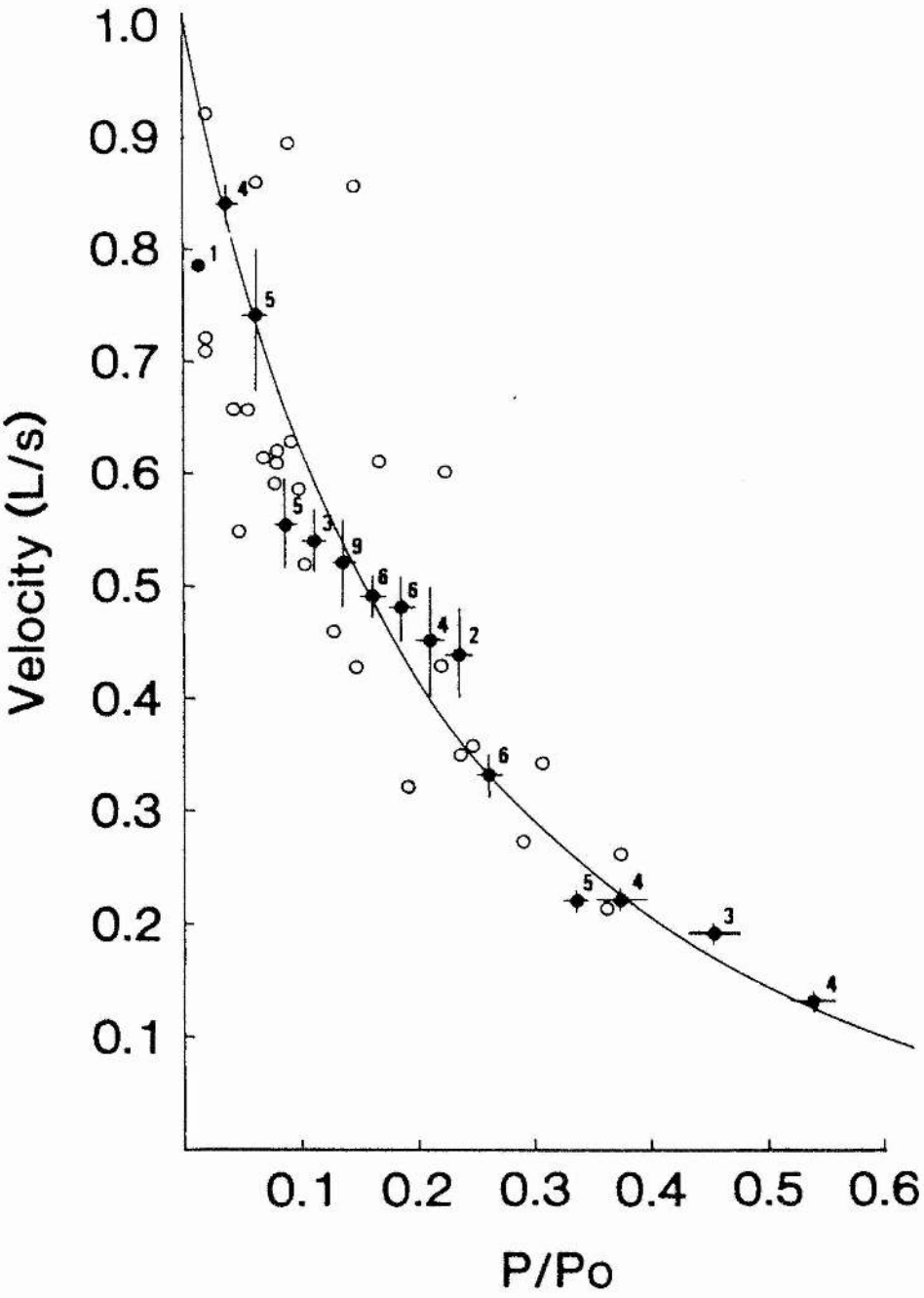

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Velocity (L/s)
0.1
0.2
0.3
0.4
0.5
0.6
P/Po

Figure 6:6: Force velocity curves from cod slow fibres. Horizontal bars indicate range of data, vertical bars ± 1 S.E., numbers = number of observations. Open circles = data from maximally activated fibres (pCa 5.18); closed circles = data from half maximally activated fibres (pCa 6.30). 8°C.

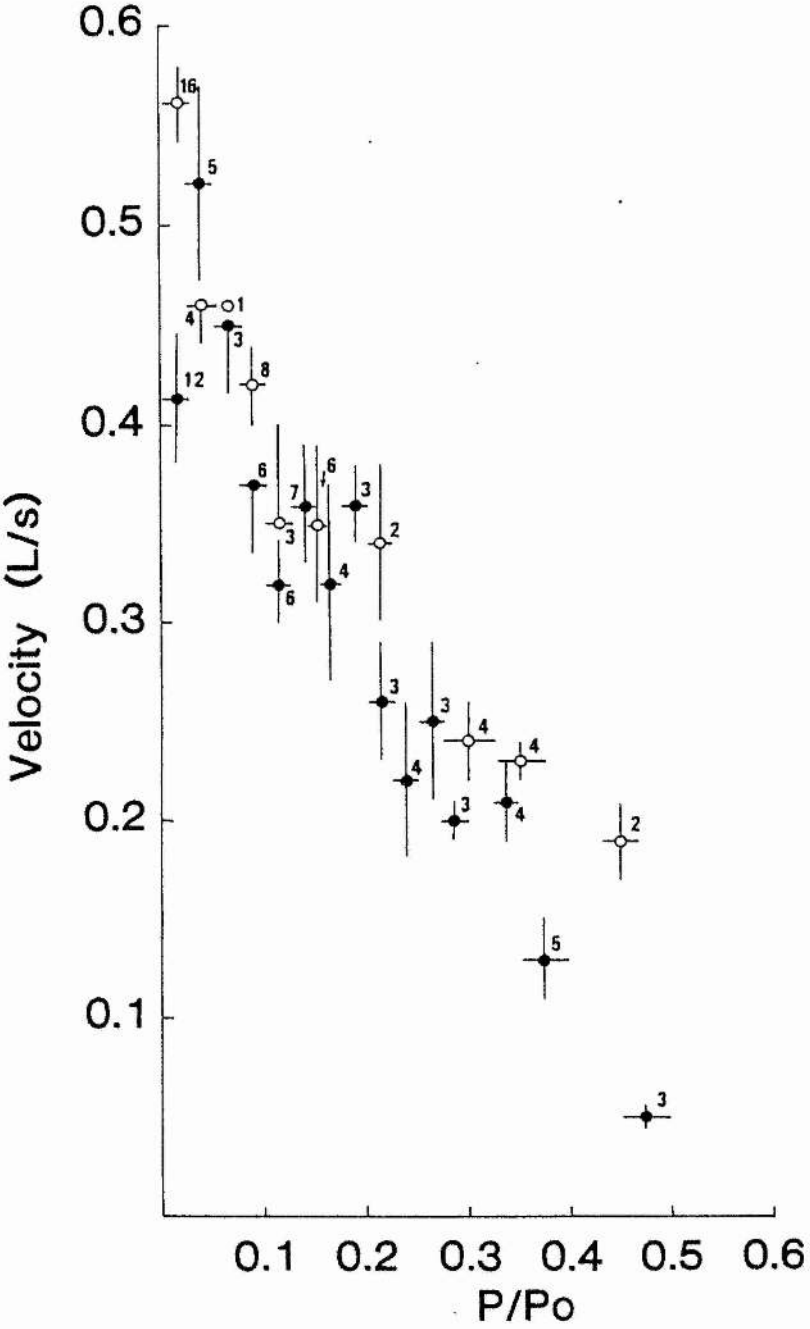

Velocity (L/s)
0.6
0.5
0.4
0.3
0.2
0.1
0.1 0.2 0.3 0.4 0.5 0.6
P/Po

Figure 6:7: Force velocity curves from dogfish myotomal muscle.

Open circles = fast fibres (3 animals, 7 fibres).

Closed circles = slow fibres (3 animals, 5 preparations).

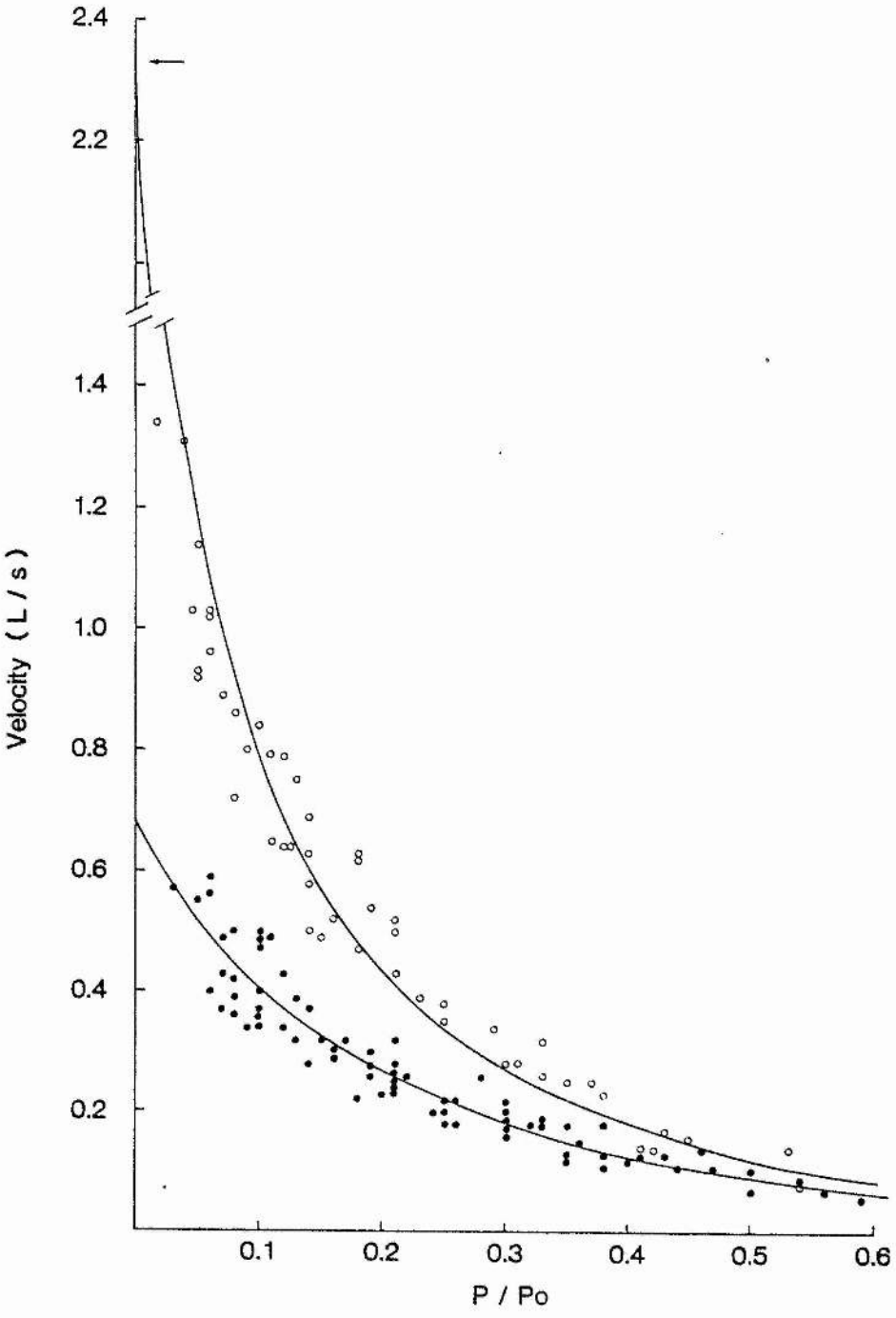

2.4
2.2
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Velocity (L / s)
0.1
0.2
0.3
0.4
0.5
0.6
P / Po

Table 6:1: Summary of V_{max} and a/P_0 for cod and dogfish muscles from the present study.

	V_{max} (L s^{-1})		a/P_0		Experimental details
	Fast muscle	Slow muscle	Fast muscle	Slow muscle	
FISH					
Cod, Gadus morhua	1.01	0.53	0.21	0.28	8°C; skinned myotomal fibres
Dogfish, Scyliorhinus canicula	2.34	0.67	0.06	0.19	8°C; skinned myotomal fibres

Table 6:2: Summary of V_{max} and a/P_0 determinations taken from the literature. References: 1 - Flitney and Johnston, 1979; 2 - Podolsky and Teichholtz, 1970; 3 - Julian, 1971; 4 - Julian and Moss, 1981; 5 - Hill, 1938; 6 - Katz, 1939; 7 - Woledge, 1968; 8 - Lannergren, 1978; 9 - Julian et al., 1981; 10 - Close, 1964; 11 - Luff, 1981.

	V_{max} (L s^{-1})		a/P_0		Experimental details
	Fast muscle	Slow muscle	Fast muscle	Slow muscle	
FISH					
Tilapia mossambica[1]	2.57	1.50	-	0.35	18°C intact fibre bundles, opercular muscles
AMPHIBIA					
Frog, Rana temporaria[2-4]	1.1 - 2.39	-	0.17 - 0.29	-	4-7°C skinned fibres, semi-tendonosus muscle
Rana temporaria[5]	1.29	-	0.26	-	0°C, whole sartorius muscle
Rana temporaria[6]	-	-	0.18 - 0.33	-	0°C, whole sartorius muscle
Anuran toad, Xenopus laevis[8]	6.34	1.10	0.38	0.10	22°C, intact single fibres iliofibularis muscle
REPTILES					
Tortoise, Testudo spp[6]	-	-	-	0.07 - 0.16	0°C, whole retractor penis muscle
Testudo spp [7]	-	0.23	-	0.07	0°C, large fibre bundle, rectus femoris muscle
MAMMALS					
Rabbit[9]	2.3	0.7	-	-	15°C EDL (fast twitch) and soleus (slow twitch) skinned fibres
Rat[10]	43 µm $sarc^{-1}$	18 µm $sarc^{-1}$	0.25	0.17	35°C whole EDL and soleus
Moose[11]	50 µm $sarc^{-1}$	23 µm $sarc^{-1}$	0.37	0.18	35°C whole EDL and soleus

Table 6:3: Contraction velocities at low loads from dogfish fast fibres incubated with and without physiological concentrations of urea (330 mM) and TMAO (180 mM). Velocity measurements listed in order experiments were performed. Temperature = 2.5°C.

Fibre	P/P_0	Velocity (Ls^{-1})	
		Standard activating solution	Activating solution + solutes
1	0.09	0.59	
	0.03		0.78
	0.03	0.80	
	0.03		0.82
2	0.06	0.86	
	0.09		0.84
	0.12	0.77	
	0.10		0.82
3	0.04	0.79	
	0.04		0.77
	0.06	0.69	
	0.07		0.69

CHAPTER 7

THE DEPENDENCE OF CONTRACTION VELOCITY ON FREE Ca^{2+} CONCENTRATION

INTRODUCTION

It is widely agreed that muscular contraction is initiated by the binding of Ca^{2+} to troponin C in vertebrate skeletal muscle (see Perry, 1979, for review). However, the mechanism underlying activation is poorly understood, and this has led to different interpretations of the exact role of Ca^{2+}. Does Ca^{2+} simply act as an on-off switch, controlling only the number of cross bridges active, and therefore force generation, or can it modulate cross bridge cycling in some way, possibly changing contraction velocity? A number of groups have studied the relationship between $[Ca^{2+}]$ and the force-velocity relationship in skinned fibres, but the results are conflicting. Podolsky and Teichholtz (1971) and Thames et al. (1974) found no change in contraction velocity at submaximal Ca^{2+} concentrations in mechanically skinned frog semitendonosus fibres. However, Julian (1971) and Julian and Moss (1981) report a significant change, a 50% decrease in V_{max} at a half maximal $[Ca^{2+}]$ in the same muscle. Wise et al. (1971) observed a similar effect in glycerinated rabbit psoas. The change in velocity at low $[Ca^{2+}]$ in the latter papers is most apparent at low loads. In the present study, emphasis has been placed on measurements at $< 0.1\ P_0$.

METHODS

Dogfish fast fibres were studied at $0.5^{o}C$ in an attempt to slow down the process of deterioration over successive activations. Each fibre was alternately maximally (pCa 5.52) and half maximally (pCa 6.39) activated, and released once against a low load when tension reached a

steady value. Velocity and tension were measured as described in Chapter 6.

RESULTS

A typical experiment from the studies on dogfish fast fibres is shown in Figure 7:1. As noted in Chapter 6, all transients show some departure from linearity, shortening velocity,generally decreasing by < 25% over the first 300 ms. Thus differences were seen in the transients obtained at pCa 6.39 relative to those from maximally activated fibres. First, the "initial" velocity (measured over the second 50 ms interval after the onset of release) was significantly lower at pCa 6.39. Secondly, the shape of the transient was dependent on $[Ca^{2+}]$. Velocity decreases at a greater rate at pCa 6.39 than at pCa 5.52. This can be seen clearly in the records of Figure 7:1 and in Figure 7:2, where the mean velocity over successive 25 ms intervals is plotted against time for the transients illustrated in Figure 7:1. This effect was clearly seen in all fibres studied, including a number of experiments performed at loads between 0.1-0.3 P_0. The "initial" velocities at pCa 5.52 and pCa 6.39 for the eight fibres studied at very low loads are shown in Figure 7:3, together with other relevant details. At least four activations were given in all experiments. In most fibres some decrease in velocity was evident over each successive pair, but the relative decrease at pCa 6.39, accompanied by the characteristic change in transient form, was always seen. The results from the initial pair of activations only have been included in these cases. A highly significant difference ($P < 0.001$) was found between the initial contraction velocities at pCa 6.39 and pCa 5.52 when analysed using a paired t test.

No decrease in velocity or change in shape of the transient was observed if a fibre was released during activation at pCa 5.52, when

the tension developed was only 0.5-0.6 P_0. This rules out the possibility of a small internal load due to inactive cross bridges retarding shortening.

A small number of experiments were performed on dogfish slow fibres, with qualitatively similar results to those obtained from fast fibres.

DISCUSSION

The Effect of Ca^{2+} on Velocity

In the present study cross bridge kinetics do appear to be dependent on $[Ca^{2+}]$. Contraction velocity over the second 50 ms interval after release is significantly lower at low $[Ca^{2+}]$. However, the step change in tension is too slow to resolve the early events, and it is not possible to say whether the velocity immediately after release is significantly different at high and low $[Ca^{2+}]$. Extrapolation of the curves in Figure 7:2 to zero time suggests that velocities at pCa 5.18 and 6.39 would be very similar. This result is typical of all other experiments. In the present study at least, the answer to the question "Is velocity dependent on $[Ca^{2+}]$?" depends upon when after the release velocity is measured. This answer does not, however, account for the different results reported by other workers who used isotonic releases on skinned fibres. Julian (1971) and Julian and Moss (1981) using faster releases (10 ms) report a significant decrease in V_{max} at low $[Ca^{2+}]$. Podolsky and Teichholtz (1970) and Thames et al. (1974) who found no effect, and Wise et al. (1971) who report an effect similar to that of Julian, measured velocity over the same interval, 50-150 ms after the onset of release. From the data presented in the above papers, it is not possible to say whether $[Ca^{2+}]$ had a significant effect on transient shape, and the subject is not discussed.

Edman (1979) studied unloaded contraction velocity in intact single frog fibres by rapid release to slack length, and measuring the time taken to take up the slack and generate tension. V_{max} was measured during tetani, twitches and twitches depressed with dantrolene, which is thought to reduce twitch height by inhibiting Ca^{2+} release from the S.R. All measurements were made within the first 30 ms after release, and V_{max} was found to be independent of the degree of activation. Costantin and Taylor (1973) used voltage clamp techniques on the sarcolemma of single frog fibres to produce graded activation. V_{max} in the first 50 ms after activation and clamping appeared to be unchanged by the level of activation.

A different approach to the problem was taken by Gulati and Podolsky (1978) who studied the very early non-linear events of the isotonic release, which are thought to reflect properties of the cross bridge mechanism (Civan and Podolsky, 1966; Huxley, 1974). These transient events were found to be insensitive to changes in $[Ca^{2+}]$, within a 10-20% uncertainty limit set by the method of analysis. It was concluded that Ca^{2+} did not affect the rate constants for cross bridge turnover.

Most of the evidence would suggest that contraction velocity, at least in the first 50 ms after release/activation, is independent of the level of activation.

The Effect of Ionic Strength on Velocity

An explanation for the results reported by Julian (1971) and Wise et al. (1971) was suggested by Thames et al. (1974), who extended the work of Podolsky and Teichholz to include studies at different ionic strengths. Both maximum isometric tension* and velocity were

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* The effect of ionic strength on P_0 has been discussed in Chapter 5.

found to be dependent upon ionic strength. At high ionic strength (> 180 mM), velocity was independent of ionic strength and $[Ca^{2+}]$. As ionic strength was decreased, velocity also decreased, and showed a marked dependence on $[Ca^{2+}]$. It was suggested that at low ionic strength, the increased resting tension observed under these conditions was due to the formation of abnormal cross bridges which retarded shortening. It was further postulated that the effect of this "internal load" could be more conspicuous in a submaximally activated fibre, explaining the Ca^{2+} dependence of contraction velocity. The ionic strength of the solutions used by Julian and Wise were less than the 180 mM used by Thames et al. A marked decrease in velocity has, however, been noted in the present study, over the same time interval measured by Thames et al., and in 180 mM solutions, in the absence of residual tensions. Subsequent experiments by Julian and Moss (1981) reinforced the earlier results of Julian (1971), and found no dependence of V_{max} on ionic strength between 90-180 mM. No explanation can be given for these conflicting observations.

Length Dependent Inactivation: The Dependence on $[Ca^{2+}]$

Some degree of non-linearity in the velocity transients can be seen in most of the work carried out on skinned fibres, but in only one paper is reference made to the phenomenon, and without discussion (Podolsky and Teichholz, 1970). However, length dependent changes in the contractile system have received attention. Edman (1975, 1976) has reported a length dependent deactivation in intact frog fibres. After a step release in length during a twitch or tetanus, the rate of redevelopment of tension, P_0, and V_{max} were decreased. The degree of deactivation was dependent on the size of the length step. The depressant effect was also found to be greater during a twitch than during a fused tetanus, suggesting that this length dependent

deactivation is in turn dependent on the level of activation, the same conclusion drawn from the present study.

Possible Mechanisms for the Observed Ca^{2+}-dependent Effects on Contraction Velocity

One possible mechanism involves Ca^{2+} binding to troponin C. Several models have been put forward to explain how the tropomyosin/troponin system regulates contraction. According to the steric hindrance hypothesis (Huxley, 1972; Haselgrove, 1972; Parry and Squire, 1973), tropomyosin is thought to physically obstruct the myosin binding site on actin at low $[Ca^{2+}]$. It is proposed that Ca^{2+} binding to troponin C produces conformational changes in troponins I and T which result in a rotation of tropomyosin in the groove between the two strands of the actin filament to expose the myosin binding sites. If this model were correct then it would be expected that at half maximal $[Ca^{2+}]$ half of the cross bridges would be free to cycle, and contraction velocity would be unaltered. However, more recent evidence is difficult to reconcile with a simple steric hindrance model.

This model predicts that when relaxed muscles go into rigor, the myosin heads push the tropomyosin into the "contraction" position. Thus, the addition of myosin heads to thin filaments under rigor conditions (low [ATP]) should reduce the binding of tropomyosin to actin. This prediction was tested by Eaton (1976) who found that myosin heads in fact induce binding between tropomyosin and actin. It was proposed that Ca^{2+} binding to troponin C resulted in structural changes in the actin monomers, induced by a cooperative effect through the troponin/troponin complex, and passed along the actin filament. A cooperative effect in the actin filament had already been proposed by Bremel and Weber (1972) to explain the observation that at low [ATP] tropomyosin enhances the binding between F actin and myosin heads. The inhibitory site on

troponin I has been identified as residues 96-117 (Syska et al., 1976), and this small peptide alone, with a molecular cut of only 2,500, is an active inhibitor. Its activity would therefore seem to be more likely to be due to a direct effect on actin than through a displacement of the very much larger tropomyosin molecule.

Further evidence against the steric hindrance model has come from structural studies, e.g. Seymour and O'Brian (1980) presented evidence to suggest that the position of tropomyosin on the thin filament precludes a direct interaction with the myosin binding sites.

The fundamental difference therefore in the models is that the actin-actin cooperativity hypothesis postulates an alteration in the myosin binding site on actin accompanying activation; one possible consequence of this is that submaximal levels of Ca^{2+} might still leave the actin free to interact with myosin, but with altered rate constants for attachment and/or detachment.

The length dependent deactivation could be explained by a decrease in the affinity of troponin C for Ca^{2+} with shortening. Evidence for such an effect in cardiac muscle has been reported by Allen and Kurihara (1981) in aequorin-loaded papillary muscles. After a rapid release, the intracellular free $[Ca^{2+}]$ (detected by changes in light emission) rose transiently, suggesting Ca^{2+} release from the thin filaments. If this were to be the case, then a dependence of velocity on the degree of activation would result in the non-linear velocity characteristics seen in the present study.

A second mechanism may involve Ca^{2+} binding to calmodulin, causing the activation of myosin light chain kinase. The latter enzyme is responsible for a Ca^{2+}-dependent phosphorylation of the P-light chain on the myosin head. While phosphorylation of myosin appears to be a prerequisite for contraction in certain smooth and invertebrate muscles, its function in vertebrate skeletal muscle is still unclear

(for review, see Bárány and Bárány, 1980). Myosin LC-kinase activity in skeletal muscle is much lower than the myofibrillar ATPase activity, and phosphorylation is therefore not in step with the cross bridge cycle. Studies with intact fibres have shown a marked increase in the phosphorylation state of myosin following a few seconds tetanus. It is possible that structural changes in the cross bridge following phosphorylation are responsible for the observed effects of Ca^{2+} on contraction velocity.

Figure 7:1: Top: Isometric activation curves for fibre 2441. Arrows indicate transfer between activating and relaxing solutions. Bottom: Isotonic releases a-d made following the above activations. 0 = zero tension; Po = maximum isometric tension; Po' = maximum tension at low $[Ca^{2+}]$; P = load. Shortening is from top to bottom.

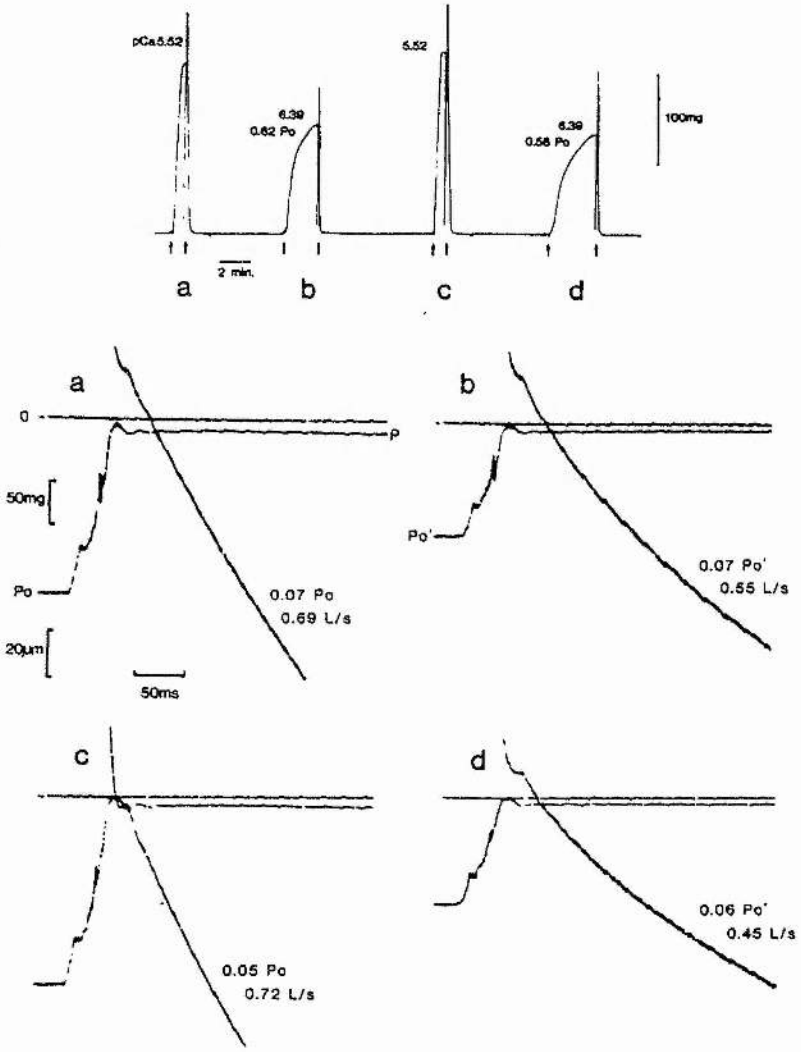
pCa5.52
6.39
0.62 Po
5.52
6.39
0.58 Po
100mg
2 min.
a
b
c
d
a
0
P
50mg
Po
0.07 Po
0.69 L/s
20µm
50ms
b
Po'
0.07 Po'
0.55 L/s
c
0.05 Po
0.72 L/s
d
0.06 Po'
0.45 L/s

Figure 7:2: Mean contraction velocity in fibre lengths s^{-1} over 25 ms intervals plotted against time. ●, ▲, ○, △ symbols represent isotonic releases a, b, c and d respectively from Figure 7:1. indicates coincidence of data from releases a and c.

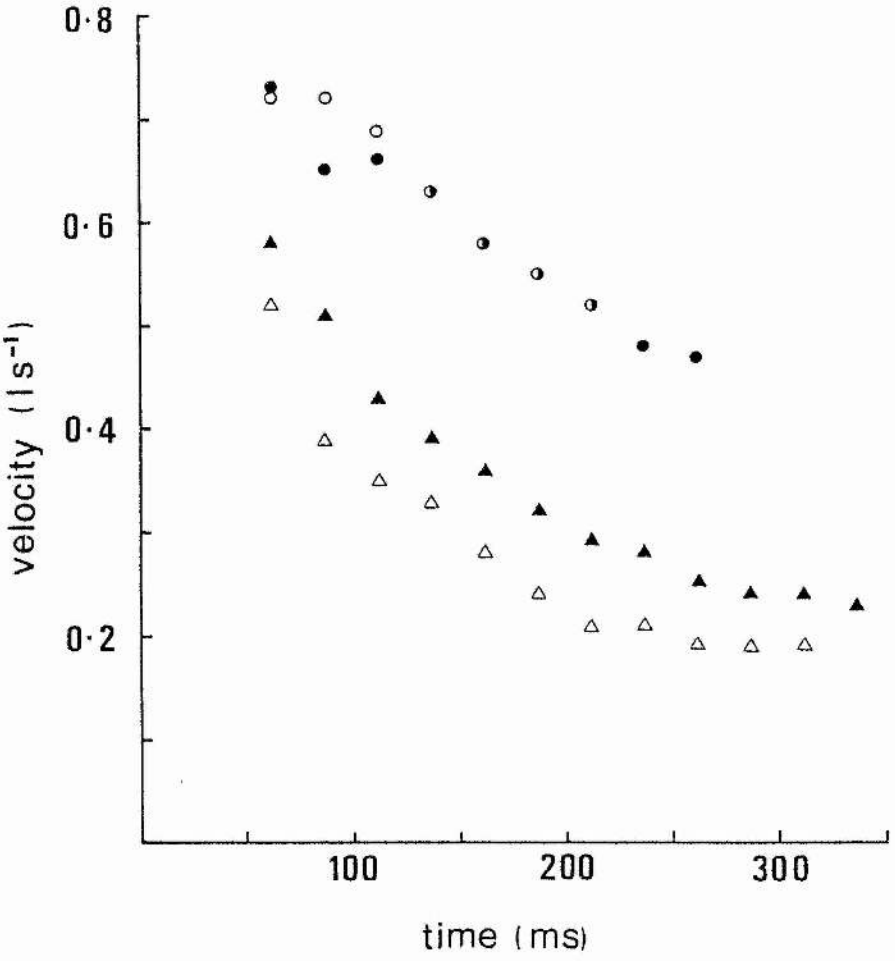
0·8
0·6
0·4
0·2
velocity (l s⁻¹)
100
200
300
time (ms)

Figure 7:3: Initial contraction velocities at maximal and submaximal $[Ca^{2+}]_{free}$ for the eight fibres studied. In most fibres, some decrease in velocity was evident over each successive pair of activations at high and low $[Ca^{2+}]$. In such cases, only the initial pair of activations have been included in the analyses. Note, however, that the relative decrease in velocity at pCa 6.39, accompanied by the characteristic change in transient form, was always seen. Errors indicate ± S.E. A paired t-test carried out on the data revealed a very significant difference ($P < 0.001$) in contraction velocity at the two PCa's studied.

Fibre	Po(6.39)/Po(5.52)	P/Po (load)	V(5.52) (L/s)	V(6.39) (L/s)	Difference
2441	0.62	0.07	0.69	0.55	0.14
	0.58	0.05	0.72	0.45	0.27
2741	0.60	0.04	0.58	0.34	0.24
0752	0.57	0.05	0.76	0.57	0.19
0753	0.63	0.06	0.66	0.46	0.20
	0.68	0.04	0.61	0.46	0.15
2051	0.53	0.07	0.80	0.67	0.13
2053	0.63	0.02	0.86	0.63	0.23
2053	0.48	0.07	0.81	0.75	0.06
2054	0.46	0.04	0.83	0.64	0.19
$\overline{X}$ ± S.E.			0.73 ± 0.03	0.55 ± 0.04	0.18 ± 0.018*
Range	0.46 - 0.68	0.02 - 0.07			

* $P < 0,001$

CHAPTER 8

GENERAL DISCUSSION

In the present study, the mechanical properties of two fish have been investigated, an elasmobranch and a teleost. There are estimated to be 25,000 living species of fish, each adapted to its own particular environment and way of life, producing an enormous variety of form and function. In the study of just these two species, many questions concerning their locomotion remain unanswered; caution must be exercised in extrapolating these results to other species. Many more species need to be studied if we are to achieve a better understanding of the mechanics and energetics of myotomal muscle in relation to locomotion.

The diversity of fish is illustrated by their adaptation to a wide range of environments. They inhabit almost every possible watery environment, from high altitude lakes, and the soda lakes of East Africa (Reite, Maloiy and Aasehaug, 1974), through fast flowing rivers, estuaries and coastal waters, to the world's oceans. Fish have also taken to dry land, for example the mudskippers, Periophthalmus, and the catfish, Clarius. Another catfish, Astroblepus marmoratus, has been seen to climb 6 m up a smooth, slightly overhanging wall using adhesion of its mouth and pelvic region. A small number have even taken to the air. The most proficient of the flying fishes, Exocoetidae, are capable of gliding up to 400 m in a single flight. Up to 14 consecutive flights have been recorded without re-entry to the water, the fish's lower caudal lobe enters the water, and by rapid tail beats (up to 70 Hz) the fish is able to accelerate for another flight (Hubbs, 1933).

Within the underwater environment, extreme conditions are met. The body temperature of most fishes closely parallels that of the environment, with a range of -2 to $+45^{\circ}C$. Species with comparable

modes of life commonly show similar metabolic rates and activity patterns over a wide range of body temperatures (see Hazel and Prosser, 1974, and Somero, 1978, for reviews). Fish also live to great depths in the ocean, where little or no light penetrates, and immense pressures are experienced. Species depth zonation is in part due to an evolutionary adaptation of the organisms proteins to pressure, and not simply to modifications in behaviour, anatomy and physiology (Somero, 1978). For example, Siebenaller and Somero (1978) found differences in pressure sensitivity in the muscle type lactate dehydrogenases of two congeneric scorpaenids living at different depths.

Body shape exhibits many forms, each showing special adaptive features. At one end of the spectrum, we have the scombroids such as the tuna, which have evolved into efficient, fast and continuous swimmers (see Magnuson, 1978). The tuna hasa laminar body form, to reduce turbulence over the surface, which produces drag and therefore wasted energy (Webb, 1975). The shape of the body and peduncle, and the high aspect ratio lunate caudal fin, minimise energy lost through drag, whilst maximising thrust, which is provided almost entirely by the caudal fin (Lighthill, 1969, 1975; Webb, 1975). In scombroids, the peduncle is very narrow, and the caudal fin is moved by the body musculature, using a system of tendons running over vertebral "pulleys" (Lindsey, 1978). Convergence towards this "thunniform" swimming mode can be seen in other groups, e.g. the sharks (Lighthill, 1969). This mode of swimming has led to complex muscle arrangements (Fierstine and Walters, 1968), which may lead to very different mechanical properties from those of other fish. At the other end of the scale, we have the undulatory (anguilliform) swimming movements of the eels, the most hydrodynamically inefficient mode, in that the inclusion of most of the body in the propulsive wave produces large vortices and cross flows, resulting in considerable drag (Lighthill, 1969). Anguilliform

locomotion is widespread, but few fish achieve high speeds and many are bottom dwellers (Lindsey, 1978). Other groups are adapted to the intricate environment of the coral reef, where manoeuvrability and the capture or evasion of prey are important. One example is the boxfishes (Ostraciidae). The body is encased in a rigid bony shell as a protection against predation, and the dorsal and anal fins are the main propulsive elements (Norman and Greenwood, 1975). Only during "rapid" swimming is the caudal fin used, by almost simultaneous contraction of all the myotomes on each side alternately (Breder, 1926). The specialized predators such as the pike (Esox lucius) have evolved elongated cylindrical bodies, a hydrodynamic adaptation for rapid acceleration (Webb, 1978). All of these various locomotory forms will impose different mechanical and energetic constraints on the musculature.

Differences in gross myotomal structure, and in fibre geometry, are found between the fish of different classes (see Bone, 1978), and these too present the possibility of different mechanical properties. Alexander (1969) has studied the arrangement of fibres in a large number of fish, and has made estimates of the degree of fibre shortening during the body flexures associated with swimming. The dogfish is an anguilliform swimmer, but the undulations are more pronounced towards the posterior, and it has a sizeable heterocereal tail. Its mode of locomotion therefore differs only slightly from the subcarangiform pattern of the cod. Alexander calculated that in the cod the fast fibres operate almost isometrically during swimming, shortening by only 2-3%. In dogfish, shortening is thought to be 7-9%, very similar to the 10% predicted for slow fibres in both fish. As discussed below, this may be of importance in a consideration of the energetics of contraction. Another interesting feature arising from Alexander's analysis was the relationship between fibre geometry and the rate of body flexure. The helical arrangement of teleost myotomal fibres gives faster bending for

a given rate of change of fibre length, at the expense of a weaker bending moment. Will thisbe reflected in the mechanical properties of fast fibres?

Differences in life-style have also led to differences in fibre types, and in the relative proportions of fast and slow fibres. Greer-Walker and Pull (1975) examined the musculature of 84 species of marine fish, in which slow fibres constituted between 0.5-29% of the myotomal muscle mass. Pelagic fish such as the Scombridae contain the highest proportion of slow fibres, and a variety of bottom dwelling and deep sea fish the lowest. Some of the Scombridae have internalised regions of slow muscle, which appear to be an adaptation to their pelagic existence. In the skipjack tuna (Kastuwonus pelamis) this internal slow muscle has been shown to have ten times as many mitochondria and a more extensive capillary supply than the superficial slow fibres (Bone, 1978a). No differences in myofibrillar ATPase activity between the deep and superficial slow fibres of the tunny (Thunnus thynnus) were found by Johnston and Tota (1974), but activities were significantly higher than in the slow muscle of the tub gurnard (Trigla lucerna), a bottom dwelling species.

As discussed in Chapter 1, fish exhibit a number of patterns of innervation and correlations have been noted between innervation and fibre recruitment at different swimming speeds (see Johnston, 1981b, for review). There is evidence to suggest that the polyneuronal fast fibre innervation of higher teleosts has evolved on eight separate occasions (Ono, D.- personal communication). The evolutionary drive for multiple innervation must be high, yet we still have no idea of the mode of activation and pattern of recruitment of such muscles. To solve this problem, it would be necessary to study the electrophysiological and mechanical properties of a nerve muscle preparation. The innervation must certainly be reflected in the contractile properties.

ON TECHNIQUES

Two models, actomyosin threads and skinned fibres, have been used in the present study to investigate the mechanical properties of fish myotomal muscle. The potential of actomyosin threads has not been realised. The results obtained were reproducible, and the technique was sensitive enough to show clear differences in the properties of actomyosins from different sources. Unfortunately it appears that the geometry and packing of the actin and myosin filaments within the threads are at least as important as the intrinsic cross bridge properties in determining their contraction velocities. For comparative studies threads would appear to be of very limited use. They may be useful in the investigation of more fundamental properties of force generation, e.g. the role of phosphorylation in contraction, since one can study specific sites of phosphorylation in isolation by manipulating the protein composition of the threads and their medium. However, care must be taken to ensure that changes in filament structure are not responsible for any changes in property observed.

The skinned fibre preparation has proven to be the more useful model. Much of the groundwork essential to the study of such a complex model has previously been done, and the technique has been useful in the study of many striated muscle systems. Skinned fibres have few of the problems of interpretation associated with actomyosin threads, since the contractile apparatus remains intact. More reliable estimates of the maximum isometric tensions generated by fish muscles have been possible, since all of the myofibrils in a preparation are active. In the polyneuronally innervated teleosts, studies with intact fibre bundles have given very low estimates of tension per unit cross sectional area. Extensive damage to the sarcolemma during dissection, resulting in a large proportion of inactive fibres, is probably

responsible for these low apparent tensions.

ENERGETIC EFFICIENCY OF FISH MUSCLES

The constant a/P_0 has been calculated for a number of fast and slow muscles (Tables 6:1, 6:2, Chapter 6). Despite large inter-species differences, a/P_0 for slow fibres is consistently smaller than that of fast fibres in all mammalian and amphibian muscles studied. The thermal and mechanical experiments performed by Woledge (1968) suggest that there is an inverse relationship between a/P_0 and efficiency (work rate/(work rate + heat rate)). The results obtained for fish in the present study differ in that a/P_0 is greater or similar in the slow muscles relative to the fast. Goldspink (1975) has shown that the chicken anterior latissimus dorsi (ADL), a true slow muscle, is 15-18 times more efficient (in terms of cost for a 1 min contraction in μmol ATP) than the fast twitch PDL during isometric contractions. However, the ADL is very inefficient under isotonic conditions. In speaking of efficiency, we must therefore consider the conditions under which the muscles operate. Alexander's (1969) estimates of the different degrees of shortening which occur during swimming in different myotomal muscles have been discussed above. Are the various myotomal fibres adapted for high isometric or high isotonic efficiency? This question points clearly to the next step in the investigation. The thermal and mechanical properties of an intact preparation must be studied simultaneously under both isometric and isotonic conditions. We can then see if the relationship between a/P_0 and efficiency described by Woledge can be extended to fish, and also determine in which mode of operation myotomal fibres are most efficient. As previously discussed, the distributed innervation of slow fibres and the fast fibres of higher teleosts makes the dissection of an intact preparation difficult.

Damaged and therefore inactive fibres will lead to errors in the heat measurements. An estimate of the proportion of inactive fibres may be obtained by comparing the tensions generated with those of maximally activated skinned fibres. The small fin and opercular muscles could provide a more suitable preparation, but they may not have the same properties as myotomal fibres. Efficiency may also be measured biochemically (Goldspink, 1975), by following the utilisation of ATP during isometric and isotonic contractions. If creatine kinase activity is inhibited with FDNB (1-fluoro-2,4-dinitrobenzene), or glycolysis/oxidative phosphorylation blocked by iodoacetate and nitrogen, the utilisation of ATP can be measured as a decrease in phosphocreatine levels.

Ca^{2+} DEPENDENT INACTIVATION

Evidence presented in Chapter 7 suggests that Ca^{2+} is involved in the modulation of a length dependent inactivation process. A length dependent inactivation process which is in turn dependent on the level of excitation has been demonstrated by Edman (1975, 1976) in live frog fibres. He suggested that deactivation was due to a decreased affinity of troponin C for Ca^{2+} with shortening. Evidence to support this has been presented by Allen and Kurihara (1981). If this is the case, as Ca^{2+} dissociates from troponin C, the proportion of attached cross bridges should decrease. Huxley and his co-workers have shown that the instantaneous stiffness of a fibre is a measure of the number of attached cross bridges. The problems in measuring this accurately are discussed in a recent paper (Huxley, 1981). The technique involves very rapid (<< 1 ms) controlled changes in length, and the measurement of the near instantaneous changes in tension. With more sophisticated apparatus than has been available for the present study, stiffness measurements

could be made at different stages of the inactivation process to monitor any change in the number of active cross bridges. The effect of changes in $[Ca^{2+}]$ on stiffness during inactivation may also be studied. The transient increase in intracellular $[Ca^{2+}]$ expected after rapid length changes could be followed by injecting the Ca^{2+} sensitive photoprotein aequorin into intact cells. The large diameter fast fibres of fish muscle are ideally suited to this technique. However, rapid transient experiments require extremely low compliance fibre attachments. Some thought must be given to this problem, since the myosepta of intact myotomal fibres could present difficulties. The method of attachment of skinned fibres may also need to be modified, e.g. small metal clamps may prove more suitable. If live fibres are to be used, those with tendonous connections could prove to be more fruitful in investigations of this phenomenon.

In conclusion, the present studies have provided us with the first accurate measurement of the isometric tensions generated by fish myotomal muscle, and the nature of the pCa-tension relation in fast and slow fibres. Force-velocity curves for both fast and slow muscles have also been derived for the first time. An interesting Ca^{2+} dependent inactivation process has been described. The physiological implications of the results are in many respects uncertain, and more work is necessary before we can achieve a better understanding of the mechanics and energetics of myotomal muscle in relation to locomotion.

APPENDIX 1

Species	$\log_{10}$ affinity constant
Imidazole - H*	7.09
EGTA - H	9.43
- 2H	18.28
- 3H	20.96
- 4H	23.04
- Mg	5.21
- MgH	12.79
- Ca	10.42
- CaH	14.75
- K	0.96
- Na	1.80
ATP - H	6.95
- 2H	10.99
- K	0.90
- Na	1.17
- Mg	4.78
- MgH	7.56
- Ca	4.40
- CaH	6.88
ADP - H	6.35
- 2H	10.34
- Ca	2.78
- Mg	3.11
- MgH	7.87
- K	1.15
- Na	1.17
Pi - H	11.89
- 2H	18.59
- 3H	20.69
- KH	12.38
- NaH	12.49
- MgH	13.77
- CaH	13.59
CP - H	2.70
- 2H	7.28
- Mg	1.60
- Ca	1.30

Affinity constants used in the iterative programme for solving ionic species binding equations.

APPENDIX 2

Teleost ringer (Hudson, 1967):

NaCl	142.2 mM
KCl	2.6 mM
$CaCl_2$	1.35 mM
$MgCl_2$	0.4 mM
$NaHCO_3$	18.5 mM
NaH_2PO_4	3.2 mM pH 7.0

Elasmobranch ringer (Meiss et al., 1974):

NaCl	134 mM
KCl	6.8 mM
$CaCl_2$	6.1 mM
$MgCl_2$	1.1 mM
Urea	208 mM
Glucose	5 mM
$NaHCO_3$	set to pH 7.4

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2. Altringham, J. D. and Johnston, I. A. (1981) J. Physiol. Lond., 310, 10-11P. The use of a moving coil galvanometer to apply isotonic loads to skinned muscle fibres.

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